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DIPEPTIDE-HYDROXAMATES ARE GOOD INHIBITORS OF THE ANGIOTENSIN I-CONVERTING ENZYME

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The inhibition constants (K_i) and modes of inhibition have been determined for a series of dipeptide-hydroxamate compounds with bovine lung parenchyma angiotensin I-converting enzyme (peptidyldipeptide carboxy-hydrolase, E.C. 3.4. 15.1). The hydroxamido function was borne by aspartic, glutamic, or aminoadipic acid and extended by 2, 3 or 4 bond lengths from the proline amide bond. L-glu(NHOH)-L-pro (K_i = 3.4 μM) and D,L-aminoadipicyl(NHOH)-L-pro(K_i = 1.2 μM) were the best competitive inhibitors of the hydrolysis of benzoyl-gly-his-gly but were not effective as affinity ligands for purification of the enzyme.

Many metallo-enzymes are dependent on zinc for their catalytic activities. Peptidyl dipeptide carboxyhydrolase (angiotensin I-converting enzyme' E.C. 3.4 15.1) contains one g atom of zinc per mol (1) and cleaves the dipeptide, histidylleucine, from the carboxyl end of angiotensin I, an inert decapeptide hormone, to produce the potent vasopressor hormone, angiotensin II (for reviews see ref 2-4). The converting enzyme also hydrolyzes bradykinin and numerous other polypeptides and N-acyl tripeptides.

The precise catalytic role of zinc in the converting enzyme is not known, but compounds that can coordinate with zinc are inhibitors of the enzyme's activity. Thus, thiol compounds (5, 6), phosphoramidates (7-9), polyphosphates (9), phosphoric and phosphonic amides (10, 11) and carboxylate compounds (12), are all good reversible inhibitors of the converting enzyme.

Nishino and Powers (13) showed that peptide-hydroxamic acids and N-acyl-N-hydroxy peptides were potent (K_i <1 μM) reversible inhibitors of thermolysin, a zinc-metallo endopeptidase. They postulated that the

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hydroxamate group formed a bidentate ligand with the active-site zinc atom (14) and subsequent X-ray crystallography studies showing that both oxygens participate in the coordination complex have proved them correct (15). Nishino and Powers (14) were able to use a peptide-hydroxamate as an affinity ligand to purify thermolysin and a neutral protease from Bacillus subtilus. Other zinc peptidases, including Pseudomonas aeruginosa elastase (16), porcine kidney leucine aminopeptidase (17) and Aeromonas proteolytica aminopeptidase (18) are inhibited by appropriate amino acid or peptide-hydroxamate compounds. 1-(3-hydroxamidopropanoyl)-L-phe was reported to be a very good (K_i = 0.2 μM)[3] inhibitor of the converting enzyme (19).

We synthesized a series of (D)- and (L)-dipeptide-hydroxamates based on C-terminal proline in which the hydroxamido moiety was borne by aspartic, glutamic, or amino adipic acid and extended by 2, 3, or 4 bond lengths from the proline amide bond. The best inhibitor is D,L-aminoadipicyl(NHOH)-L-pro (K_i = 1.2 μM) and with all the inhibitors that we tested, the -NHOH derivative was more potent than the corresponding $-OCH_3$ derivative.

MATERIALS AND METHODS

Enzyme source and purification

Bovine lung converting enzyme was purified as we described previously (20, 21) with the following modifications:

(1) After the affinity chromatography step, the fractions containing the enzyme were pooled, and concentrated 10-fold by ultrafiltration (PM-10 ultrafiltration membrane, Amicon Corp.) and then applied to a 3 x 30 cm column of AcA-22 Ultrogel (LKB) equilibrated in 1mM Tris pH 8.3.

(2) The pooled enzyme fractions were again concentrated by ultrafiltration and applied to a 2.5 x 20 cm column of hydroxlapatite (Bio-Rad) equilibrated in 1mM Tris, pH 8.3. The void volume fractions containing the enzyme were pooled and lyophilized.

The homogeneous enzyme that we obtain (~2mg per 500 g of lung tissue) has a specific activity of 12-14 units/mg.

Enzyme Assay

Converting enzyme activity was routinely measured fluorometrically by hydrolysis of Bz-gly-his-leu[4] (Sigma) as originally described by Cushman and Cheung (22) and as we detailed previously (6, 23). For detailed kinetic studies, including determination of all kinetic constrants, we used Bz-gly-his-gly as substrate because it shows much less substrate inhibition[5]. The method of assay, however remains the same.

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3. Calculated from the I_{50} and K_m valves given in the cited reference.
4. Abbreviations used: BOC, tert-butyloxycarbonyl; Bz, benzoyl; Bzl, benzyl; Tris, tris (hydroxymethyl) aminomethane.
5. Ohlsson, J.T., and Wilson, I.B., unpublished observations.

Bz-gly-his-gly was prepared from benzoyl chloride and gly-his-gly (24). In our assay buffer (6), the K_m of Bz-gly-his-gly is 3mM.

High Performance Liquid Chromatography

HPLC was used to demonstrate purity of protected amino acids and synthesized peptides. The conditions for HPLC were as we described previously (21).

Synthesis of peptide-hydroxamates

The dipeptides used in this study were synthesized by conventional procedures (24, 25) using dicylclohexylcarbodiimide (Sigma) as the coupling reagent. Pro-OBzl was obtained from Sigma and the β-, γ-, and δ-methyl ester derivatives of (D)- and (L)-Asp (Sigma), (D)- and (L)-Glu(Sigma), and D,L-aminoadipic acid (Chemalog) were prepared using $SOCl_2$ in methanol (24). The BOC derivatives of the esterified amino acids were prepared by reaction with di-tert-butyldicarbonate (Pierce) (26). The BOC- and -OBzl protecting groups were removed in saturated HBr/acetic acid and the hydrobromide salts of the monoesterified dipeptides were precipitated overnight at 4°C with anhydrous ether. The corresponding hydroxamate derivatives were prepared from the esterified dipeptides in 0.5M NH_2OH (at pH>11) and a portion of each esterified dipeptide was also saponified in NaOH. At every step of the synthesis, the number of amino group equivalents were determined by ninhydrin assay (24) and the number of ester group equivalents by hydroxamic acid colorimetric assay (27). The purity of the products was assessed by thin layer chromatography in three different solvent systems and by HPLC. The composition of each dipeptide was quantitatively confirmed by amino acid analysis of the 20h acid hydrolysate (28).

The overall synthetic yield of each dipeptide was: L-asp(OCH_3)-L-pro (54%, 8.4 mmol, 2.7g); L-asp(NHOH)-L-pro (54%, 8.1 mmol, 2.6g); L-asp-L-pro (49%, 0.27 mmol, 0.83g); D-asp (OCH_3)-L-pro (14%, 1.2 mmol, 0.4g); D-asp(NHOH)-L-pro (14%, 1.1 mmol, 0.36 g); D-asp-L-pro (11%, 0.079 mmol, 0.024g); L-glu(OCH_3)-L-pro (64%, 2.4 mmol, 0.81 g); L-glu(NHOH)-L-pro (64%, 2.3 mmol, 0.78 g); L-glu-L-pro (45%, 0.070 mmol, 0.022g); D-glu(OCH_3)-L-pro (71%, 4.9 mmol, 1.7 g); D-glu (NHOH)-L-pro (71%, 4.7 mmol, 1.6 g): D-glu-L-pro (65%, 0.183 mmol, 0.060 g); D,L-aminoadipicyl(OCH_3)-L-pro (70%, 0.7 mmol, 0.25g); D,L-aminoadipicyl(NHOH)-L-pro (70%, 0.5 mmol, 0.18g); D,L-aminoadipicyl-L-pro (53%, 0.15 mmol, 0.05g).

Kinetic Studies

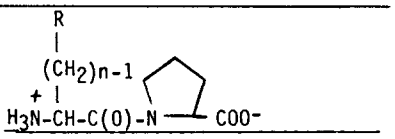
The dipeptides were tested as inhibitors of converting enzyme activity using double reciprocal Lineweaver-Burk plots of v^{-1} vs $(S)^{-1}$. Bz-gly-his-gly was the substrate and several inhibitor concentrations around the value of the K_i were used.

RESULTS AND DISCUSSION

Based on the structure of captopril (1-(2-D-methyl-3-mercaptopropanoyl)-L-pro) (5) and D-cys-L-pro(6), we synthesized first D-Asp(NHOH)-L-pro as a potential inhibitor of the converting enzyme. Pro was chosen because other investigators (4,5) showed that it interacts very well with the secondary binding site (S_2')[6] of the enzyme. D-Asp was chosen because its side chain should be long enough to allow interaction between the -NHOH moiety of the inhibitor and the active-site zinc atom. D-Asp(NHOH)-L-pro is a strong competitive inhibitor of the enzyme (Table 1). The effectiveness of

6. The nomenclature used for the individual amino acids of an inhibitor (P_1, P_1', P_2') and for the binding subsites of the enzyme (S_1, S_1', S_2', etc) is that of Schecter and Berger (29).

Table I: K_i values for inhibition of converting enzyme with dicarboxylate-dipeptide derivatives[a]



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n	Amino Acid	-C(O)NHOH	-C(O)O⁻	-C(O)OCH₃
2	D-Asp	7	14	550
2	L-Asp	10	58	350
3	D-Glu	8	7	73
3	L-Glu	3.4	14	49
4	D,L-Aminoadipicyl	1.2[b]	28	110

[a]all compounds tested showed competitive inhibition of converting enzyme activity. The K_i values are μM determined with Bz-gly-his-gly as the substrate in 30mM Tris-300mM NaCl buffer containing Na_2SO_4, pH 8.3. These values are the average of the results using at least 3 different concentrations of inhibitor.

[b]the better diasterioisomer probably has $K_i \geq 0.6$ μM and $K_i \leq 1.2$ μM.

inhibition appears to depend on the length of the P_1 amino acid side chain; L-Glu(NHOH)-L-pro was a slightly better inhibitor than D-Asp(NHOH)-L-pro and D,L-aminoadipicyl(NHOH)-L-pro was the best inhibitor (K_i = 1.2 μM) that we tested. Hydroxylamine (up to 10mM) had no effect on enzyme activity.

The (D)- and (L)-amino acid-dipeptide hydroxamates have similar potencies. This is in contrast to captopril (5) or D-cys-L-pro (6), where the D-derivatives are 30 and 10 fold stronger inhibitors than the L-derivatives.

We also examined inhibition by the dicarboxylate dipeptides (Table 1). Other investigators (30) found that straight chain carboxylate-Pro compounds of the general structure, $HOOC-(CH_2)_n-C(O)$-L-pro (n = 0, 1, 2, 4) were poor inhibitors of the converting enzyme, (K_i = 113 - 1640 μM)[3] but when n = 3, the K_i = 24 μM. Adding a D-methyl group at the C2 position gave Ki = 7.5 μM (n=2) and K_i = 1.7 μM (n=3). Our results suggest that a D-amino group at the C2 position is approximately equivalent to a D-methyl group for binding to the enzyme.

The K_i values determined for the inhibitors were the same using impure preparations or homogeneous preparations of enzyme. In each case, inhibition was not progressive with time and was reversible by dilution or dialysis.

We prepared α-N-(acetyl)-D-Asp-L-Pro and D-Asp-L-Pro(OBzl) and found that acetylating the α-amino group ruined the binding about 60-fold (K_i = 830 μM and esterifying the prolyl carboxyl group ruined the binding by 11-fold (K_i = 160 μM). We attempted to prepare an affinity gel for the converting enzyme and immobilized L-glu(NHOH)-L-pro and D,L-aminodipicyl(NHOH)-L-pro through their prolyl-carboxyl groups or through their terminal amino groups to Sepharose CL-6B substituted either with diaminohexane or succinylated ethylenediamine, repectively, but the gels did not specifically bind the enzyme.

In general, our results reflect the better zinc binding properties of hydroxamates as compared to carboxylate compounds. The best hydroxamate is at least six times more potent than the best carboxylate derivative. The carboxylate-esters, which can coordinate zinc only very weakly, are much poorer inhibitors (Table 1).

The compounds that we have described bind at the active-site of the enzyme and appear to coordinate with zinc. The converting enzyme thus falls into the group of zinc proteases that are inhibited strongly by hydroxamate peptides.

REFERENCES

1. Das, M. and Soffer, R.L. (1975) J. Biol. Chem. 250, 6762-6768.
2. Soffer, R.L. (1976) Ann. Rev. Biochem. 45, 73-94.
3. Swales, J.D. (1979) Pharmacol. Ther. 7, 173-201.
4. Ondetti, M.A., and Cushman, D.W. (1982) Ann. Rev. Biochem. 51, 283-308.
5. Cushman, D.W., Cheung, H.S., Sabo, E.F., and Ondetti, M.A. (1977) Biochemistry 16, 5484-5491.
6. Harris, R.B., Ohlsson, J.T., and Wilson, I.B. (1981) Arch. Biochem. Biophys. 206, 105-112.
7. Holmquist, B., and Vallee, B.L. (1979) Proc. Nat'l Acad. Sci. 76, 6216-6220.
8. Galardy, R.E. (1980) Biochem. Biophys. Res. Comm. 97, 94-99.
9. Galardy, R.E. (1982) Biochemistry 21, 5777-5781.
10. Thorsett, E.D., Harris, E.E. Peterson, E.R., Greenlee, W.J., Patchett, A.A., Ulm, E.H., and Vassil, J.C. (1982) Proc. Nat'l. Acad. Sci. 79, 2176-2180.
11. Galardy, R.E., Kontoyiannidou-Ostrem, V., and Kortylewicz, Z. (1983) Biochemistry 22, 1990-1995.

12. Patchett, A.A., Harris, E.E., Trustrom, E.W., Wyvratt, M.J., Wee, M.T., Taub, D., Peterson, E.R., Iheler, T.J., tenBrocke, J., Payne, L.G., Ondeykay, D.L., Thorsett, E.D., Greenlee, W.J., Lohr, N.S., Hoffsommer, R.D., Joshua, H., Ruyle, W.V., Rothrock, J.W., Aszer, S.D., Maycock, A.L., Robinson, F.M., Hirshman, R., Sweet, C.S., Ulm , E.H., Gross, D.M., Vassil, T.C., and Stone, C.A. (1980) Nature (London) 288, 280-283.
13. Nishino, N. and Powers, J.C. (1978) Biochemistry, 17, 2846-2850.
14. Nishino, N., and Powers, J.C. (1979) Biochemistry 18, 4340-4347.
15. Holmes, M.A., and Matthews, B.W. (1981) Biochemistry 20, 6912-6920.
16. Nishino, N., and Powers, J.C. (1980) J. Biol. Chem. 255, 3482-3486.
17. Chan, William W.-C., Dennis, P., Demmer, W., and Brand, K. (1982) J. Biol. Chem. 257, 7955-7957.
18. Baker, J.O., Wikes, S.H., Bayliss, M.E., and Prescott, J.M. (1983) Biochemistry (1983) 22, 2098-2103.
19. Ondetti, M.A., Sabo, E.F., Losee, K.R., Cheung, H.S., Cushman, D.W., and Rubin, B. (1977) in: Peptides: Proc. 5th Am. Peptide Symp. Goodman, M., and Meinhofer, J., eds. John Wiley, N.Y. pp 576-578.
20. Harris, R.B., and Wilson, I.B. (1982) Int'l. J. Pep. Prot. Res. 20, 167-176.
21. Harris, R.B., and Wilson, I.B. (1983) J. Biol. Chem. 258, 1357-1362.
22. Cushman, D.W., and Cheung, H.S. (1971) Biochem. Pharmacol. 20, 1637-1648.
23. Harris, R.B., Ohlsson, J.T., and Wilson, I.B. (1981) Anal. Biochem. 45, 78-94.
24. Greenstein, J.D., and Winitz, M. (1961) in Chemistry of the Amino Acids. Vol. 2 John Wiley and Sons, New York.
25. Voelter, W. and Schmid-Siegmann, E. (1983) in Peptides: Syntheses-Physical Data, Vol. 2. Addison-Wesley ublishing Co., Mass.
26. Moroder, L., Hallett, A., Wunsch, E., Keller, O., and Wersin, G. (1976) Hoppe-Zeylers, Z. Physiol. Chem. 357, 1651-1653.
27. Cheronis, N.D., and Entricker, J.B. (1963) in Identification of Organic Compounds, John Wiley and Sons, New York, p. 140.
28. Spackman, I.H., Stein, W.H., and Moore, S. (1958) Anal. Chem. 30, 1190-1206.
29. Schecter, I., and Berger, A. (1967) Biochem. Biophys. Res. Comm. 27, 157-161.
30. Cushman, D.W., and Ondetti, M.A. (1980) Prog. Med. Chem. 17, 42-104.