JOURNAL OF
MEDICINAL
CHEMISTRY

© Copyright 1989 by the American Chemical Society

Volume 32, Number 4 April 1989

Communications to the Editor

Benzofurans as Mechanism-Based Inhibitors of Dopamine β-Hydroxylase

Sir:

Dopamine β-hydroxylase (DβH; EC 1.14.17.1), a copper-containing monooxygenase, catalyzes the hydroxylation of dopamine to form norepinephrine. DβH exhibits a wide substrate specificity and has been shown to hydroxylate many compounds including benzyl cyanides,[1-3] phenyl thioethers,[4] phenethylamines,[5] and phenylpropenes.[6] Many of these alternate substrates are also mechanism-based inhibitors of DβH[8] and have been used as probes of the enzyme's catalytic mechanism[8,9] and these data have recently been summarized.[7] The mechanism by which DβH activates molecular oxygen to a species that is capable of carrying out insertion into a carbon–hydrogen bond,[5] oxidation at sulfur,[4] and epoxidation of an olefin[10] is still not well understood. A better understanding of the reaction mechanism of DβH should help in the designing of tight binding inhibitors that could be antihypertensive agents.

Compounds containing olefinic functional groups were shown to be effective mechanism-based inhibitors of DβH.[10-12] May et al.[12] showed that the binding of aromatic olefinic inhibitors is very sensitive to the addition of bulky substituents at the β-olefinic position. Thus, cinnamylamine was a poor mechanism-based inhibitor of DβH with an immeasurably high K_i, whereas α-methylstyrenes had K_i values ranging from 0.52 to 5.2 mM.[10] These workers suggested a mechanism involving the intermediacy of a copper oxygen species that catalyzes removal of an electron from the olefin to generate a radical cation intermediate.

In a continuation of our investigation of mechanism-based inhibitors of DβH, we have synthesized a series of straight chain (**1**, **2**) and bicyclic olefinic substrates (**3–5**) and determined the inactivation kinetics for each compound (Table I). The bicyclic analogues **3–5** were designed to have reduced steric hindrance at the β olefinic carbon. In addition the bicyclic compounds are capable of producing a radical cation intermediate with the potential for an increased lifetime and thus a greater probability of reaction with an active-site nucleophile. The net effect for the inactivation reaction would be a decrease in the partition ratio, i.e., the ratio of the number of turnovers per inactivation event.

All inhibitors (**1–5**) studied exhibited pseudo-first-order inactivation, and the rate of inactivation was shown to be dependent on the oxygen concentration. The presence of the substrate tyramine protected DβH from inactivation. Taken together these results indicate that reduced DβH, oxygen, and inhibitor (**1–5**) were required for inactivation. However, the compounds can be divided into two classes distinguished by their requirement for ascorbate as a concomitant reductant in the inactivation reaction. *p*-Hydroxystyrene (**1**) and *trans*-3-(*p*-hydroxyphenyl)-2-propene (**2**) required ascorbate for inactivation; 5-, 6-, or 7-hydroxybenzofuran (**3–5**) did not require ascorbate for inactivation. Also, it was shown that in the absence of ascorbate and in the presence of inhibitor **4**, DβH catalyzes conversion of tyramine to octopamine. Under these conditions no loss of activity was observed, indicating tyramine protected DβH from inactivation and benzofurans **3–5** were solely reductants. Indeed, enzyme-bound Cu(II) could be

(1) Colombo, G.; Giedroc, D. P.; Rajashekhar, B.; Villafranca, J. J. *J. Biol. Chem.* **1984**, *259*, 1601–1606.
(2) Colombo, G.; Rajashekhar, B.; Ash, D. E.; Villafranca, J. J. *J. Biol. Chem.* **1984**, *259*, 1607–1615.
(3) Colombo, G.; Rajashekhar, B.; Giedroc, D. P.; Villafranca, J. J. *J. Biol. Chem.* **1984**, *259*, 1593–1600.
(4) May, S. W.; Phillips, R. S.; Mueller, P. W.; Herman, H. H. *J. Biol. Chem.* **1981**, *256*, 8470–8475.
(5) Klinman, J. P.; Krueger, M. *Biochemistry* **1982**, *21*, 67–75.
(6) Rajashekhar, B.; Fitzpatrick, P. F.; Colombo, G.; Villafranca, J. J. *J. Biol. Chem.* **1984**, *259*, 6925–6930.
(7) Fitzpatrick, P. F.; Villafranca, J. J. *Arch. Biochem. Biophys.* **1987**, *257*, 231–250.
(8) Fitzpatrick, P. F.; Villafranca, J. J. *J. Am. Chem. Soc.* **1985**, *107*, 5022–5023.
(9) Bossard, M. J.; Klinman, J. P. *J. Biol. Chem.* **1986**, *261*, 16421–16427.
(10) Padgette, S. R.; Wimalasena, K.; Herman, H. H.; Sirimanne, S. R.; May, S. W. *Biochemistry* **1985**, *24*, 5826–5839.
(11) Bargar, T. M; Broersma, R. J.; Creemer, L. C.; McCarthy, J. R.; Hornsperger, J.-M.; Palfreyman, M. G.; Wagner, J.; Jung, M. J. *J. Med. Chem.* **1986**, *29*, 315–317.
(12) May, S. W.; Mueller, P. W.; Padgette, S. R.; Herman, H. H.; Phillips, R. S. *Biochem. Biophys. Res. Commun.* **1983**, *110*, 161–168.

0022-2623/89/1832-0735$01.50/0 © 1989 American Chemical Society

Table I. Kinetic Parameters of DβH Inactivation by Inhibitors 1–5

no.		R_1	K_i,[a] mM	k_{inact},[b] min^{-1}	partition[b] ratio
1[c]	R_1	H	0.70 ± 0.07	0.025 ± 0.001	730 ± 50
2[c]	R_1	CH_3	>20		680 ± 50

no.	R_2	R_3	R_4	K_i,[a] mM	k_{inact},[b] min	partition ratio[b]
3[d]	OH	H	H	1.90 ± 0.80	0.23 ± 0.06	8 ± 4
4[d]	H	OH	H	0.04 ± 0.01	0.23 ± 0.02	27 ± 3
5[d]	H	H	OH	3.2 ± 0.20	0.25 ± 0.01	35 ± 3

[a] In a typical inactivation experiment 20–40 μg of bovine DβH, purified by the method of Colombo et al.,[16] was incubated in solutions of 0–3% DMF incubated at 25 °C containing 100 mM Mes, 100 mM NaOAc, pH 5.0, 0.5 mg/mL catalase, 12 mM ascorbate, and varied concentrations of inhibitor in a total volume of 200 μL. At time intervals enzyme activity was determined by removing an aliquot from the incubation mixture (10–20 μL) and diluting into a 3-mL solution of assay mix at 37 °C containing 100 mM NaOAc, pH 5.5, 30 mM tyramine, 12 mM ascorbate, and 0.017 mg/mL catalase and then measuring oxygen consumption. To obtain linear kinetics for 6-hydroxybenzofuran (4) only 2 μg of DβH could be added to the preincubation mix to prevent over consumption of 4. This level of enzyme is too low for use with the oxygraph, necessitating the use of a stopped assay. In a procedure adapted from Feilchenfeld et al.,[17] 50-μL aliquots were removed from the inactivation solution and diluted into 450 μL of assay mix which was stirred and incubated at 37 °C for 15 min, and the reaction was stopped by the addition of 100 μL of 2 N HCl. Activity was determined by chromatography of a 50-μL aliquot of the assay mix on a C_{18} column with heptanesulfonic acid as an ion-pairing reagent and monitored at 224 nm. The octopamine peak was integrated and the concentration of octopamine was calculated from a predetermined standard curve of octopamine. $k_{inact\ apparent}$ was determined by plotting the natural logarithm of specific activity remaining versus time for each concentration of inhibitor. The slope of each linear regression fit represents the $k_{inact\ apparent}$ for the concentration of inhibitor. Replots of $1/k_{inact\ apparent}$ versus 1/[I] were fit with Cleland's hypero program to determine k_{inact} and K_i. [b] Partition ratios were determined by quantitating the amount of inhibitors 1–5 consumed after complete inactivation of a known amount of DβH.[18] A typical reaction mixture contained 15–20 μg of bovine DβH, 0.5 mg/mL catalase, 20 μM 6-hydroxybenzofuran (4), 12 mM ascorbate, 125 mM Mes, 125 mM NaOAc, pH 5.5, in a total volume of 0.5 mL; inactivations were done at 25 °C. Concentrations of inhibitors 1–5 were determined by chromatography of an aliquot (50 μL) on a Waters C-18 μBondapak with water–acetonitrile; the absorbance was monitored at a UV maximum of each inhibitor (1-5). [c] 1 and 2 were previously synthesized by Dale and Hennis[19] and Spath and Bruck,[20] respectively. [d] 3–5 were previously synthesized by René et al.[21]

reduced by the benzofurans as detected by monitoring the decrease in the ESR signal of Cu(II).

When DβH was partially inactivated with compounds 1–5, extensive dialysis did not result in restoration of activity, suggesting that the active site has been covalently modified. Thus, compounds 1–5 all fall into the category of mechanism-based inhibitors.[13] The kinetic parameters at a single oxygen concentration for inactivation of DβH by 1–5 are given in Table I.

Analysis of the data in Table I led to the following conclusions. When a β-methyl group was added to the β

(13) Walsh, C. T. *Tetrahedron* **1982**, *38*, 871–909.

Scheme I. Proposed Mechanism of Inactivation for 5-Hydroxybenzofuran, Involving a Radical Cation Intermediate and Showing Partitioning between Inactivation (Pathway a) Involving Nucleophilic Attack by an Active-Site Amino Acid or Collapse to Products (Pathway b) To Form a Postulated Epoxide[10]

olefinic carbon of *p*-hydroxystyrene (1), a large increase in the K_i was found, suggesting steric hindrance of the binding of *trans*-3-(*p*-hydroxyphenyl)-2-propene (2) at the active site. Eliminating rotation of the benzene ring with respect to the benzylic position of *p*-hydroxystyrene (1) in the series of benzofurans 3–5 had a variable effect on the K_i values, reducing by nearly 20-fold the K_i for compound 4 but increasing the value for compounds 3 and 5. Clearly the steric influence of the β-methyl substituents is directly related to K_i whereas a contribution from rotation of the benzene ring about the benzylic position of *p*-hydroxystyrene is unimportant. For the bicyclic compounds 3–5, the rate of inactivation is 9–10 times faster than for *p*-hydroxystyrene, suggesting that the planar relationship between the olefinic double bond and the benzene ring provides the optimum "conformation" for inactivation by these compounds.

When the hydroxyl group is at the 5 (3) or 7 (5) position in the benzofurans, poorer binding is found, indicating the position para to the olefinic bond elicits the tightest binding; however, there is no effect on the kinetic rate of inactivation for any of these benzofurans.

Partition ratios (turnover/inactivation) were measured by following the disappearance of the olefin substrates 1–5 with time.[16] After complete inactivation of DβH, the amount of each olefinic substrate 1–5 consumed was determined. No decrease in the amount of 1–5 was observed in the absence of DβH. The partition ratios of *p*-hydroxystyrene (1) and *trans*-3-(*p*-hydroxyphenyl)-2-propene (2) are virtually identical, suggesting a partitioning

(14) Kruse, L. I.; Kaiser, C.; De Wolf, W. E., Jr.; Chambers, P. A.; Goodhart, P. J.; Ezekiel, M.; Ohlstein, E. H. *J. Med. Chem.* **1988**, *31*, 704–706.
(15) Bargar, T. M.; Broersma, R. J.; Creemer, L. C.; McCarthy, J. R.; Horsperger, J.-M.; Attwood, P. V.; Jung, M. J. *J. Am. Chem. Soc.* **1988**, *110*, 2975–2976.
(16) Colombo, G.; Papadopoulos, N. J.; Ash, D. E.; Villafranca, J. J. *Arch. Biochem. Biophys.* **1987**, *252*, 71–80.
(17) Feilchefeld, N. B.; Richter, H. W.; Waddell, W. H. *Anal. Biochem.* **1982**, *122*, 124–128.
(18) Waley, S. G. *Biochem. J.* **1985**, *227*, 843–849.
(19) Dale, W. J.; Hennis, H. E. *J. Am. Chem. Soc.* **1958**, *80*, 3645–3649.
(20) Spath, E.; Bruck, J. *Ber.* **1938**, *71*, 2708–2711. Spath, E. *Manatsh.* **1914**, *35*, 326.
(21) René, L.; Royer, R. *Bull. Soc. Chim. Fr.* **1973**, *7–8*, 2355–2356.

Table II. Comparison of Kinetic Parameters of Previously Published Mechanism-Based Inhibitors (6–8) of DβH

no.	structure	K_i, mM	k_{inact}, min^{-1}	k_{inact}/K_i (M^{-1} min^{-1})
6		0.52[a]	0.81[a]	1560
7		0.057[b]	0.184[b]	3230
4		0.040	0.23	5750
8		0.005[c]	0.15[c]	30000

[a] Apparent values at pH 5.0, 0.24 mM O_2, 37 °C.[10] [b] Apparent values at pH 5.0, 0.24 mM O_2, 37 °C.[14] [c] Apparent values at pH 5.0, 0.24 mM O_2, 37 °C.[15]

of a similar intermediate along the reaction pathway. With 6-hydroxybenzofuran (**4**), a 27-fold decrease in the partition ratio was found as compared to that of **1**, whereas 5-hydroxybenzofuran (**3**), which binds with a K_i larger than that of *p*-hydroxystyrene (**1**), exhibits a 90-fold lower partition ratio.

A substantial change in the partition ratio may be explained by increased resonance stabilization of the radical cation intermediate. The net effect of this added stabilization would be to lower the transition state for formation of the intermediate and also to increase the probability of trapping the resonance-stabilized intermediate by an enzyme nucleophile (Scheme I). The oxygen in the furan ring of benzofurans **3–5** can serve to stabilize the radical cation as an oxycation radical (shown in Scheme I). The radical cation of 6-hydroxybenzofuran (**4**) can also be further stabilized by loss of a proton from the 6-hydroxyl group to form a "quinone-like" radical intermediate. Michael addition of an active-site nucleophile to the radical cation intermediate would result in the formation of a stable adduct at the active site of DβH. The difference in the partition ratio of benzofurans **3** and **4** could be either due to the difference in the positioning of these molecules in the active site or due to a difference in the ability of the enzyme to stabilize the radical cation intermediates during catalysis.

Table II provides a comparison of kinetic constants of 6-hydroxybenzofuran (**4**) versus several other published mechanism-based inhibitors. The k_{inact} for **4** was determined at 25 °C versus 37 °C for **6–8**[10,14,15] and may be a factor of 2 larger at 37 °C. Even so the bicyclic analogue **4** is clearly a potent mechanism-based inhibitor of DβH on the basis of the criterion of a large k_{inact}/K_i value.[15]

In summary each of the bicyclic olefins (**3–5**) is able to stabilize a radical cation intermediate more effectively than *p*-hydroxystyrene (**1**) through resonance forms involving oxycations or other delocalized radical intermediates. This stabilization would potentially increase the life time of the reactive intermediate, leading to more effective enzyme inactivation. This added stabilization of the radical cation intermediate could be the explanation for the large decrease in the partition ratio with these analogues compared to those of the simple olefins **1** and **2**.

Acknowledgment. Portions of this work were supported by the National Institutes of Health Grant GM29139.

Registry No. **1**, 2628-17-3; **2**, 20649-39-2; **3**, 13196-10-6; **4**, 13196-11-7; **5**, 4790-81-2; DβH, 9013-38-1.

* To whom correspondence should be addressed.

G. King Farrington, Alok Kumar
Joseph J. Villafranca*

Department of Chemistry
The Pennsylvania State University
University Park, Pennsylvania 16802
Received August 22, 1988

Carboxyalkyl Dipeptides with Atrial Natriuretic Factor Potentiating and Antihypertensive Activity

Sir:

The atrial natriuretic factors (ANF) are peptide hormones with powerful vasodilatory, diuretic, and natriuretic effects.[1] Much work has been done toward establishing their physiological and pathophysiological roles in blood pressure regulation and fluid-volume and electrolyte homeostasis.[2] Recent reports[3] demonstrating the ability of neutral endopeptidase (NEP) (EC 3.4.24.11) to inactivate ANF in vitro prompt us to report our work on NEP inhibitors that potentiate the hypotensive activity of exogenous ANF and express antihypertensive activity.

Peptide hormones are usually eliminated in various ways: uptake by target tissues and intracellular degradation, inactivation by blood proteases or by the liver or by the kidneys. Although the metabolism of ANF is not completely understood, studies to date indicate that ANF may be metabolized within the kidney, liver, lung, and vasculature.[4,5] The kidney is one of the target organs where the activity of ANF is expressed. Major among the brush border peptidases of the kidney proximal tubule is NEP, a zinc-containing protease that cleaves peptide bonds on the amino side of the residues with hydrophobic side chains.[6] However, NEP has a heterogeneous distribution and has been found in a variety of tissues.[7] Thus, we speculated that NEP, in the kidney and elsewhere, might be important in the degradation of ANF in vivo. We hypothesized that inhibition of this enzyme could modulate the in vivo activity of both exogenously administered as well as endogenously generated ANF. After the initiation of this project, several publications disclosed that ANF is a substrate for NEP and that the enzyme rapidly inactivates this peptide in vitro.[3] These results are consistent with our hypothesis.

In order to test this hypothesis, we required compounds that are potent and selective inhibitors of NEP, since inhibition of related metallopeptidases, such as angiotensin

(1) de Bold, A. J.; Borenstein, H. B.; Veress, A. T.; Sonnenberg, H. *Life Sci.* **1981**, *28*, 89. Currie, M. G.; Geller, D. M.; Cole, B. R.; Boylan, J. G.; Sheng, W. Y.; Holmberg, S. W.; Needleman, P. *Science* **1983**, *221*, 71.
(2) Genest, G.; Cantin, M. *Rev. Physiol. Biochem. Pharmacol.* **1988**, *110*, 1.
(3) Stephenson, S. L.; Kenny, A. J. *Biochem. J.* **1987**, *243*, 183. Kenny, A. J.; Stephenson, S. L. *FEBS Lett.* **1988**, *232*, 1 and references cited therein.
(4) Espiner, E. A.; Crozier, I. G.; Nicholls, M. G.; Cuneo, R.; Yandle, T. G.; Ikram, H. *Lancet* **1985**, 398.
(5) Luft, F. C.; Lang, R. E.; Aronoff, G. R.; Ruskoaho, H.; Toth, M.; Ganten, D.; Sterzel, R. B.; Unger, T. *J. Pharmacol. Exp. Ther.* **1986**, *236*, 416.
(6) Kenny, A. J.; Maroux, S. *Physiol. Rev.* **1982**, *62*, 91.
(7) Gee, N. S.; Bowes, M. A.; Buck, P.; Kenny, A. J. *Biochem. J.* **1985**, *228*, 119.

 © 1989 American Chemical Society