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INACTIVATION OF TYROSINE HYDROXYLASE BY REDUCED PTERINS

Donald M. Kuhn and Walter Lovenberg

Section on Biochemical Pharmacology, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, Maryland 20205

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ABSTRACT: Tyrosine hydroxylase [E.C. 1.14.16.2] is inactivated by incubation with its reduced pterin cofactors L-erythro-tetrahydrobiopterin, 2-amino-4-hydroxy-6-methyl-5,6,7,8-tetrahydropterin and 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropterin. Each of the two diastereoisomers of L-erythro-tetrahydrobiopterin inactivates tyrosine hydroxylase but the natural (6R) form is much more potent than the unnatural (6S) form at equimolar concentrations. The pterin analog 6-methyl-5-deazatetrahydropterin, which has no cofactor activity, also inactivates the enzyme whereas the oxidized pterins 7,8 dihydrobiopterin and biopterin do not. The inactivation process is both temperature and time dependent and results in a reduction of the V_{max} for both tetrahydrobiopterin and tyrosine. Neither tyrosine nor oxygen inactivates tyrosine hydroxylase.

Tyrosine, tryptophan and phenylalanine hydroxylases are pterin-dependent monooxygenases and represent a family of enzymes with many common characteristics as well as distinctive properties (1). In the case of phenylalanine hydroxylase, exposure of the enzyme to its substrate phenylalanine *in vitro* results in marked increases in catalytic activity (2,3,4). Such a mechanism may have a physiologic role by increasing the capacity of the liver to metabolize phenylalanine when excess substrate is present in tissue. This phenomenon has not been observed with the other pterin-dependent enzymes, probably because they do not serve a major catabolic role for their respective substrates. It is likely that regulatory mechanisms for tyrosine and tryptophan hydroxylase have evolved based on the need for the products of the metabolic pathways that they initiate, i.e., catecholamines and serotonin, respectively.

Since prior work in our laboratory suggested that tetrahydrobiopterin (BH_4)[1] may play a key role in regulating the endogenous activity of tyrosine

[1] Abbreviations: BH_4, L-erytho-tetrahydrobiopterin; $6MPH_4$, 2-amino-4-hydroxy-6-methyl-5,6,7,8-tetrahydropterin; $DMPH_4$, 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropterin; BH_2, 7,8-dihydrobiopterin.

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hydroxylase, we examined the interaction of this and related pterins with the enzyme. Exposure of highly purified tyrosine hydroxylase to BH_4 results in marked destabilization of the enzyme and a marked increase in its heat lability. This destabilization does not occur when the other cosubstrates are present and the enzyme is executing catalysis.

MATERIALS AND METHODS: L-3,5-^{3}H-tyrosine (43 Ci/mmol) was purchased from Amersham. L-tyrosine, dithiothreitol, $6MPH_4$, $DMPH_4$, and catalase were obtained from Calbiochem. Biopterin, BH_4 and BH_2 were purchased from Dr. B. Shirks, Postfach, CH-8623 Wetzikon, Switzerland. The (6R) and (6S) diastereoisomers of BH_4 were generously provided by Dr. Michael Parniak. The 6-methyl-5-deazatetrahydropterin was a gift from Dr. Stephen Benkovic and 2,5,6-triamino-4-pyrimidinone 2 HCl was provided by Dr. June Ayling. Tissue culture media was purchased from GIBCO Laboratories.

Tyrosine hydroxylase was purified from cultured rat pheochromocytoma (PC12) cells. PC12 cells were maintained in 150 cm^2 tissue culture flasks in a medium consisting of Dulbecco's modified Eagle Medium supplemented with 10% horse serum, 5% fatal calf serum, 100 units/ml penicillin and 90 ug/ml streptomycin. Cells were grown at 37°C in an atmosphere of 95% air and 5% CO_2. Medium was changed twice weekly and cells were harvested from the flasks by mechanical agitation. Tyrosine hydroxylase was purified from PC12 cells and was judged to be homogenous by SDS polyacrylamide gel electrophoresis. The method of purification will be communicated in detail elsewhere. The specific activity of the enzyme was 130 nmol/mg/min. Tyrosine hydroxylase was assayed by the tritium release method (5). Assays were always carried out with saturating concentrations of BH_4 so that total residual activity was measured. Anaerobic incubations were carried out as previously described (6). Protein was measured by the method of Bradford (7).

RESULTS: Preincubation of purified tyrosine hydroxylase with its natural cofactor results in a loss of catalytic activity. The results shown in Fig. 1 (left panel) indicate that BH_4 inactivates tyrosine hydroxylase in a concentration and time-dependent manner. Tyrosine does not cause inactivation nor does oxygen since control enzyme activity did not vary from that shown in Fig. 1 under anaerobic incubation (data not shown). The BH_4 inactivation of tyrosine hydroxylase was also temperature dependent as shown in Fig. 1 (right panel). Control enzyme was relatively stable up to 37°C whereas enzyme incubated with BH_4 loses activity more rapidly with increasing temperatures. In fact, the BH_4 effect was evident at 4°C. Kinetic analysis revealed that the V_{max} of tyrosine hydroxylase was reduced after preincubation with BH_4 to the same extent as was total activity (50%) whereas the K_m values of the inactivated enzyme for tyrosine and BH_4 were not changed (data not shown).

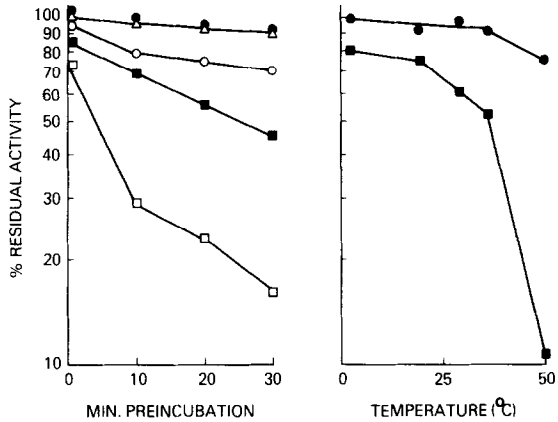

Fig. 1. Effects of preincubation of tyrosine hydroxylase with BH_4. Left panel: tyrosine hydroxylase (160 µg/ml) in 10 mM sodium phosphate buffer, pH 7.2, containing 5% sucrose (w/v) and 1.0 mM EDTA was mixed with BH_4 at the indicated final concentrations and placed in a water bath at 37°C. Preincubations were carried out for the times indicated on the abscissa after which a 10 µl sample of the enzyme was transferred to a tyrosine hydroxylase reaction mixture at 4°C. Residual enzyme activity was subsequently assayed at 37°C for 15 min at saturating concentrations of BH_4 (1.0 mM). The symbols are control (●), 0.25 mM BH_4 (○), 0.50 mM BH_4 (■), 1.0 mM BH_4 (□), and 0.1 mM tyrosine (△). Right Panel: Effects of preincubation temperature on the inactivation of tyrosine hydroxylase by BH_4. Tyrosine hydroxylase was mixed with 10 mM HCl or 0.5 mM BH_4 and incubated for 30 min at the temperatures indicated on the abscissa. After preincubation, all samples were chilled to 4°C and assayed for residual tyrosine hydroxylase as described above. The symbols are control (●) and BH_4 (□). All pterins were prepared in 0.01 N HCl and standardized spectrophotometrically immediately before use.

Chemical reduction of L-erythro-BH_4 produces two diastereoisomers referred to as the (6R)- and (6S)-forms (8). Experiments with each isomer, which had been resolved from the (6R,S) mixture by reverse-phase high performance liquid chromatography, revealed that the natural (6R)-BH_4 form was much more potent than both the unnatural (6S)-BH_4 form and the (6R,S)-BH_4 mixture at equimolar concentrations. These data are shown in Fig. 2.

A variety of other pterins were tested for their ability to inactivate tyrosine hydroxylase and these results are summarized in Table 1. The synthetic pterins $6MPH_4$ and $DMPH_4$ inactivated the enzyme much like BH_4 but these compounds were somewhat less potent than the natural cofactor. The pterin analog 6-methyl-5-deazatetrahydropterin and the pyrimidine 2,5,6-triamino-4-pyrimidinone also caused inactivation of tyrosine hydroxylase. These latter compounds have little, if any, cofactor activity with tyrosine hydroxylase but each can bind to the hydroxylase as judged by their ability to competitively

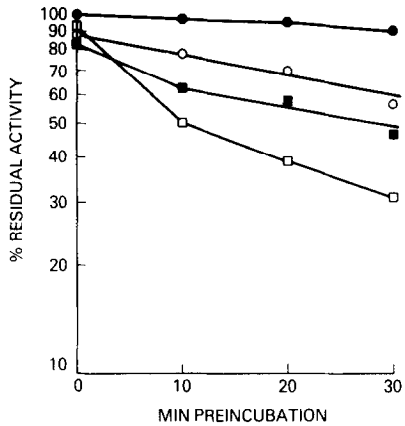

Fig. 2. Effects of preincubation with BH_4 diastereoisomers on tyrosine hydroxylase. Enzyme was mixed with 10 mM HCl (●), or with 0.5 mM concentrations of 6S (○), 6R,S (■), or 6R (□) BH_4 and incubated at 37°C for 30 min. Samples were chilled to 4°C and 10 µl aliquots were subsequently assayed for residual tyrosine hydroxylase as described in Fig. 1. The diastereoisomers were prepared fresh in 0.01 N HCl and standardized spectrophotometrically prior to use.

inhibit BH_4 cofactor activity (9,10). Finally, BH_2 and biopterin, the oxidized forms of BH_4, were without effect on tyrosine hydroxylase.

Since the non-enzymatic oxidation of BH_4 can generate peroxides which inhibit tyrosine hydroxylase, a variety of radical scavengers and reducing agents were tested for their ability to protect the enzyme from BH_4 inactivation. Ferrous ions were also tested since loss of iron from tyrosine hydroxylase reduces activity (11). The data in Table 2 indicate that catalase, dithiothreitol, and ferrous ions were ineffective in preventing tyrosine hydroxylase inactivation by BH_4. These same reagents are components of the enzyme assay so it is apparent that the inactivation of tyrosine hydroxylase is not reversed by these agents.

DISCUSSION: In the current study, we have shown that highly purified tyrosine hydroxylase rapidly loses catalytic activity when exposed to BH_4 in the absence of the cosubstrate tyrosine. This destabilization appears to be independent of the third substrate molecular oxygen. It appears likely that this loss of activity results from the interaction of the reduced pterin with the enzyme at the normal pterin binding site on the enzyme. In studies done

Table 1. Inactivation of tyrosine hydroxylase by reduced pterins.

Pterin	Concentration (mM)	% Residual Activity
Control	--	91
BH_4	0.50	52
$6MPH_4$	1.00	27
$DMPH_4$	0.50	61
6-methyl-5 deaza-tetrahydropterin	0.50	57
2,5,6-triamino-pyrimidinone	1.00	44
BH_2	0.50	84
biopterin	0.50	85

Tyrosine hydroxylase was mixed with the pterins at the final concentrations listed and incubated at 37°C for 30 min. After preincubation, samples were chilled to 4°C and assayed for residual tyrosine hydroxylase activity as described in Fig. 1. All compounds except BH_2 were prepared in 0.01 N HCl just prior to use. BH_2 was dissolved in potassium phosphate buffer, pH 7.4, and standardized spectrophotometrically.

with relatively crude tyrosine hydroxylase preparations (12), concentration dependencies indicated that BH_4 was more effective than $6MPH_4$ or $DMPH_4$ in stimulating loss of activity. In addition, as seen in Figure 2, the natural 6R form of BH_4 was more effective than the 6S isomer, once again suggesting specificity of binding. Since 6-methyl-5-deazatetrahydropterin can inactivate

Table 2. Effects of catalase, dithiothreitol, and ferrous ions on the inactivation of tyrosine hydroxylase by BH_4.

Addition	Concentration	% Residual Activity
none	--	53
catalase	100 µg/ml	52
dithiothreitol	2.0 mM	48
Fe^{2+}	50 µM	57

BH_4 was added to tyrosine hydroxylase at a concentration of 0.5 mM and the indicated additions were made just prior to placing the enzyme solutions in a 37°C water bath for 30 min. After preincubation, samples were chilled to 4°C and assayed for residual tyrosine hydroxylase activity as described in Fig. 1.

tyrosine hydroxylase but has little substrate activity, this result suggests that pterin binding to the enzyme and not cofactor activity, per se, underlies the pterin-induced inactivation of tyrosine hydroxylase.

The mechanism of destabilization cannot be determined by the experiments to date. One obvious possibility is that a reduced oxygen species or radical is formed during interaction of the enzyme with BH_4. This would appear not to be the mechanism of inactivation for the following reasons: 1) The inactivation occurs in the absence of molecular oxygen; 2) catalase, ferrous ions, and dithiothreitol had no effect upon the inactivation; 3) 6 methyl-5-deazatetrahydropterin which is not enzymatically oxidized (9) and therefore would not generate reduced oxygen species is effective in destabilizing the enzyme. A more likely possibility is that the reduced pterin exerts a direct effect on the enzyme. The reaction mechanism of tyrosine hydroxylase appears to be random sequential (10). Thus, if the enzyme normally accepts electrons from the electron donor during catalysis, incubation with a reduced pterin in the absence of tyrosine would result in the enzyme being largely in the reduced state. In turn, the enzyme may be more succeptible to thermal inactivation when it is reduced. It is of interest to note that recent studies have also suggested that tyrosine hydroxylase is more labile after it has been phosphorylated and is in an activated state (13,14,15). While the physiological relevance of BH_4 inactivation of tyrosine hydroxylase is unknown, this phenomenon may represent a basic mechanism by which the enzyme responds to its substrates. Preliminary evidence from our laboratory and others (16) indicates that BH_4, in lower concentrations than presently used, enhances the destabilizing influence of phosphorylation on tyrosine hydroxylase. Perhaps many *in vitro* treatments which activate tyrosine hydroxylase by reducing the K_m for BH_4 will also lead to increased lability. For example, phosphatidylinositol both activates and inactivates tyrosine hydroxylase (17).

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REFERENCES

1. Kaufman, S. (1974) In Aromatic Amino Acids in the Brain, pp. 85-108, Elsevier, New York.
2. Ayling, J. R. and Helfand, G. D. (1975) In Chemistry and Biology of Pteridines, pp. 305-319, De Gruyter, Berlin.
3. Shiman, R. and Gray, D. W. (1980) J. Biol. Chem. 255, 4793-4800.
4. Kaufman, S. and Mason, K. (1982) J. Biol. Chem. 257, 14667-14678.
5. Lerner, P., Nose, P., Ames, M. M. and Lovenberg, W. (1978) Neurochem. Res. 3, 641-651.
6. Kuhn, D. M., Ruskin, B. and Lovenberg, W. (1980) J. Biol. Chem. 255, 4137-4143.
7. Bradford, M. M. (1976) Anal. Biochem. 72, 248-254.
8. Bailey, S. W. and Ayling, J. E. (1978) J. Biol. Chem. 253, 1598-1605.
9. Moad, G., Luthy, C. L., Benkovic, P. A. and Benkovic, S. J. (1979) J. Amer. Chem. Soc. 101, 6068-6076.
10. Bullard, W. P. and Capson, T. L. (1983) Mol. Pharmacol. 23, 104-111.
11. Hoeldtke, R. and Kaufman, S. (1977) J. Biol. Chem. 252, 3160-3169.
12. Kuhn, D. M. (1983) Trans. Amer. Soc. Neurochem. 14, 175.
13. Lazar, M. A., Truscott, R. J. W., Raese, J. D. and Barchas, J. D. (1981) J. Neurochem. 36, 677-682.
14. Vrana, K. E., Alhiser, C. L. and Roskoski, R. (1981) J. Neurochem. 36, 92-100.
15. Vrana, K. E. and Roskoski, R. (1983) J. Neurochem. 40, 1692-1700.
16. Roskoski, R., Finney, T. P., Tate, D. J. and Vrana, K. E. (1983) Fed. Proc. 42, 2022.
17. Lloyd, T. (1979) J. Biol. Chem. 254, 7247-7254.