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Inactivation of Tyrosine Hydroxylase by Pterin Substrates Following Phosphorylation by Cyclic AMP-Dependent Protein Kinase

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SUMMARY

We reported previously that, following phosphorylation by cyclic AMP-dependent protein kinase, tyrosine hydroxylase in rat corpus striatal extracts is inactivated in a time-dependent and apparently irreversible fashion. Removal of low molecular weight substances from these extracts by gel filtration attenuates this inactivation. We tried to determine the identity of endogenous metabolites that promote inactivation of tyrosine hydroxylase under our experimental conditions. In the present study, we report that the reducing co-substrate tetrahydrobiopterin and its analogues promoted this irreversible inactivation. The concentration that produced a 50% loss of activity (at 20 min) of the phosphorylated enzyme was 0.7 μM and that for the unphosphorylated enzyme was 420 μM. Using enzyme purified from a rat pheochromocytoma, we found that tyrosine, α-methyl-*p*-tyrosine, and a 3-iodotyrosine protected the phosphorylated enzyme against the inactivation produced by tetrahydrobiopterin. Catecholamines (dopamine, norepinephrine, epinephrine, and some of their analogues) also nullified inactivation. In contrast, the product of the reaction, dihydroxyphenylalanine, failed to attenuate the inactivation process. We performed several studies to ascertain the mechanism of inhibition by tetrahydrobiopterin. We considered the possibility that it formed reactive free radicals that produced inhibition. Free radical scavengers, however, failed to block the inhibition produced by tetrahydrobiopterin. Superoxide dismutase, catalase, and peroxidase also failed to protect tyrosine hydroxylase against inactivation. Moreover, when the experiments were performed under anaerobic conditions, the inactivation process was unaffected. These results suggest that reactive oxygenated species were not required for inactivation by tetrahydrobiopterin.

Tyrosine hydroxylase (EC 1.14.16.2) catalyzes the rate-limiting reaction in the biosynthesis of catecholamines (1). Kaufman (2) identified BH_4 as the naturally occurring cofactor for phenylalanine hydroxylase in liver. This facilitated the characterization of tyrosine hydroxylase, which also requires this compound as an obligatory reducing co-substrate (2–4). Tryptophan hydroxylase also utilizes this compound as substrate (see Ref. 5). These three aromatic amino acid hydroxylases constitute a family of enzymes that exhibit homologous protein structures, as deduced from cDNA nucleotide sequence analysis (6–8).

Tyrosine hydroxylase is a substrate for cAMP-dependent protein kinase *in vitro* (9, 10). Following phosphorylation, the K_m of the enzyme for its reducing tetrahydropterin co-substrate is decreased as the enzyme is activated (10). Extensive studies indicate that the enzyme is phosphorylated and activated under physiological conditions (see Ref. 11). The natural co-substrate for tyrosine hydroxylase is (6*R*)-BH_4 (12). Analogues including (6*RS*)-6MPH_4 and $DMPH_4$ have been commonly used as synthetic substrates for activity determinations. The stoichiometry of the reaction catalyzed by tyrosine hydroxylase is given by the following chemical equation (12).

$$\text{Tyrosine} + BH_4 + O_2 \rightarrow \text{DOPA} + \text{q-}BH_2 + H_2O$$

The concentration of BH_4 in rat adrenal is about 12 μM (13) and that in rat corpus striatum is about 2 μM (14). Because these values are subsaturating at pH 7 and because phosphorylation by cyclic AMP-dependent protein kinase decreases the apparent K_m (15, 16), the availability of reducing co-substrate is likely to play a role in the regulation of tyrosine hydroxylase activity (see Ref. 12).

We previously reported that, after activation by cyclic AMP-dependent phosphorylation, tyrosine hydroxylase in corpus striatal homogenates loses its activity in a time-dependent and irreversible fashion (17). Following the removal of low molecular weight substances by gel filtration, the inactivation of the phosphorylated enzyme is greatly attenuated. Addition of frac-

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ABBREVIATIONS: BH_4, 5,6,7,8-tetrahydro-L-biopterin; $DMPH_4$, 6,7-dimethyl-5,6,7,8-tetrahydropterin; 6-MPH_4, 6-methyl-5,6,7,8-tetrahydropterin; PIPES, piperazine-*N*,*N*′-bis(α-ethanesulfonic acid); q-BH_2, quinonoid-7,8-dihydrobiopterin; DOPA, dihydroxyphenylalanine; BH_2, 7,8-dihydrobiopterin.

tions containing the low molecular weight substances to the enzyme under phosphorylation conditions results in the return of the inactivation phenomenon (17). Ascorbate is one agent that promotes inactivation, with an EC_{50} of 6 μM at pH 6.0 (18). We previously reported, in preliminary form (19), that reducing co-substrates such as 6-MPH_4 also promoted inactivation of tyrosine hydroxylase under phosphorylating conditions. Here we present additional experiments on the inactivation of tyrosine hydroxylase by reducing co-substrates.

Materials and Methods

Enzyme source and activity determinations. Gel-filtered corpus striatal homogenates were prepared as previously described (17, 18). Tyrosine hydroxylase was purified from a transplantable rat pheochromocytoma to a specific activity of approximately 0.5 μmol/min/mg of protein (pH 6, 1.0 mM 6-MPH_4, 30°), as documented previously (10, 20). The enzyme was >95% pure, as judged by polyacrylamide gel electrophoresis (18). Cyclic AMP-dependent protein kinase was purified to homogeneity from bovine heart by procedures previously described (21). Tyrosine hydroxylase was assayed radiometrically by a coupled decarboxylase procedure with 100 μM tyrosine and subsaturating (125 μM) 6-MPH_4, as previously documented (22), at pH 6.0 unless noted otherwise. Protein was measured by the procedure of Bradford (23), using bovine γ-globulin as standard.

Phosphorylation of tyrosine hydroxylase by cyclic AMP-dependent protein kinase. The phosphorylation of gel-filtered enzyme from corpus striatum and inactivation by pterins were performed concomitantly during a 20-min incubation (18). Portions (10 μl) were then taken for tyrosine hydroxylase activity measurements. The determinations of the EC_{50} values were performed as previously reported (18).

Studies with purified tyrosine hydroxylase required additional manipulations. The phosphorylation of tyrosine hydroxylase purified from pheochromocytoma was performed with 100 μM ATP, 10 mM $MgCl_2$, 50 mM PIPES (pH 7.0), and 40 μg/ml catalytic subunit of cyclic AMP-dependent protein kinase, during a 10-min incubation at 30° (20), and the mixture (250 μl) was gel filtered at 4° with Sephadex G-15 (1 × 20 cm) to remove ATP. The tyrosine hydroxylase and protein kinase were concentrated by pressure dialysis to protein concentrations of 0.5–1.5 mg/ml and were stored at −70°. The phosphorylated enzyme was stable for more than 3 months and exhibited 3- to 5-fold greater activity than nonphosphorylated enzyme when assayed at pH 6.0. The specific activity, however, decreased 5–10% with each freeze-thaw cycle. The inactivation processes in each experiment were studied with the same lot of enzyme and the standard error was within 10% of the reported value. The filtration and storage buffer consisted of 30 mM potassium phosphate and 50 mM NaF at pH 7.0. This buffer was chosen to inhibit any adventitious protein phosphatase activity (24). The stoichiometry of ^{32}P incorporation, determined as previously described (20), ranged from 0.65 to 0.99 mol of phosphate/mol of tyrosine hydroxylase subunit among the various preparations. The incorporation of less than 1 mol suggests that the isolated enzyme contained endogenous phosphate, as directly demonstrated by Nelson and Kaufman (25) for the bovine adrenal and rat corpus striatal enzyme.

Inactivation reactions. Phosphorylated or untreated tyrosine hydroxylase (20 μg/ml) was incubated with the final specified concentration of BH_4 in 25 mM PIPES at 37° for 20 min, in the presence or absence of the specified compound, in a total volume of 100 μl. All inactivation reactions were performed at pH 6.0 unless otherwise noted. The compound whose stabilizing or protective effect was being assessed was added 1–2 min before the addition of BH_4. After the 20-min incubation, the mixtures were gel filtered at 4° with Sephadex G-15 (0.9 × 10 cm) in 30 mM potassium phosphate (pH 6.0), 50 mM NaF, after incubation to remove the low molecular weight compounds, whose presence might alter catalytic activity. The enzyme-containing fractions were assayed immediately or stored in buffer containing 0.5 mg/ml bovine serum albumin, at −70°, before assay. In specified experiments where the additions did not alter enzyme activity, this gel filtration following the inactivation procedure was not performed and the samples were stored at −70° with 0.5 mg/ml of added bovine serum albumin.

Materials. D-Mannitol was purchased from Pfanstiehl Laboratories, Inc. (Waukegan, IL). L-[^{14}C]Tyrosine was purchased from ICN (Irvine, CA). (6*R*)-BH_4, (6*RS*)-6-MPH_4, and the corresponding dihydro derivatives were purchased from Dr. B. Schirks Laboratories (Jona, Switzerland). Stock solutions of these pterins were freshly prepared in 5 mM HCl for each experiment. The concentrations were determined spectrophotometrically using the wavelengths, pH, and absorbencies given by Pfleiderer (26). Bovine γ-globulin was purchased from Bio-Rad (Richmond, CA). The other chemicals and enzymes were obtained from Sigma Chemical Co. (St. Louis, MO).

Results

General characterization of the inactivation of tyrosine hydroxylase by pterins. We previously showed that striatal tyrosine hydroxylase is irreversibly inactivated by reduced pterins under phosphorylation conditions (19). For the present studies, it was necessary to characterize the extent of activation by the catalytic subunit of cyclic AMP-dependent protein kinase and the stability of the activated gel-filtered enzyme. Addition of purified bovine cardiac muscle catalytic subunit at a final concentration of 1 μM (40 μg/ml) produced about a 12-fold activation of gel-filtered striatal tyrosine hydroxylase activity (Fig. 1). The enzyme retained 80% or more of its maximum activity during a 20-min preincubation under phosphorylation conditions. 6-MPH_4, however, produced a concentration- and time-dependent decrease in activity under phosphorylation conditions. For example, incubation with 0.1 and 1 μM 6-MPH_4 produced a 40 and 80% decrease in activity following a 20-min incubation (Fig. 1). Based on these results, we examined the concentration dependence for inactivation by a variety of agents during a standard 20-min preincubation at 37°.

Our initial studies showed that 6-MPH_4 inactivated the phosphorylated but not the nonphosphorylated enzyme (19). Kuhn and Lovenberg (27), on the other hand, reported that inactivation occurred under nonphosphorylation conditions using enzyme prepared from PC12 cells. We, therefore, performed more complete concentration dependence studies for inactivation under both conditions. Nonphosphorylated tyrosine hy-

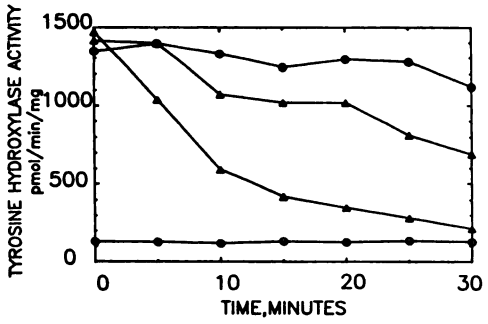

Fig. 1. Inactivation of striatal tyrosine hydroxylase by 6-MPH_4 under phosphorylation conditions. Gel-filtered corpus striatal extracts were incubated at 37° for the time indicated and samples were removed and immediately assayed for tyrosine hydroxylase activity with 125 μM 6-MPH_4 as described in Materials and Methods. ○, Nonphosphorylation; ●, phosphorylation; △, phosphorylation plus 0.1 μM 6-MPH_4; ▲, phosphorylation plus 1 μM 6-MPH_4. Data from one experiment are given. Similar results were obtained in five other experiments.

droxylase was inactivated by 6-MPH_4, but this inactivation was associated with an EC_{50} more than 3000-fold greater (Fig. 2). Computer analysis of the data (28, 29) indicated that the EC_{50} values for 6-MPH_4 were 2.2×10^{-7} M and 6.9×10^{-4} M for phosphorylated and nonphosphorylated enzyme, respectively.

We next examined a variety of pterins to determine their relative effectiveness in mediating inactivation of the phosphorylated and nonphosphorylated enzyme. Like 6-MPH_4, the natural co-substrate (BH_4) was more effective in mediating enzyme inactivation under phosphorylation conditions than in the absence of ATP and protein kinase catalytic subunit (Table 1). Another widely used synthetic co-substrate, $DMPH_4$, also mediated inactivation. The reduced pterin analogue 2,5,6-triamino-4-hydroxypyrimidine also inactivated phosphorylated tyrosine hydroxylase (Table 1). Most of the inactivation reactions were performed at pH 6.0, because this is the optimum pH of the native enzyme (12). We found that the EC_{50} values for 6-MPH_4 and BH_4 were 2–3 times greater at pH 7.2 than at pH 6.0 (Table 1). Nevertheless, these compounds were potent inhibitors at pH 7.2.

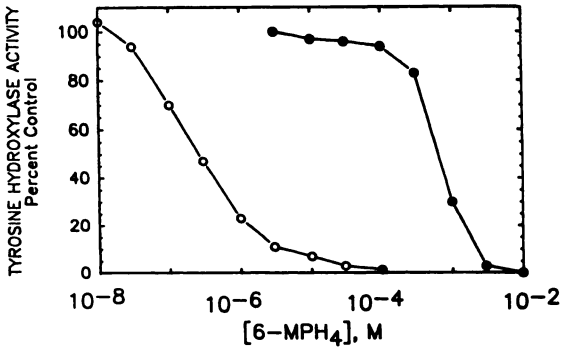

Fig. 2. Tyrosine hydroxylase activity is more sensitive to inactivation by 6-MPH_4 under phosphorylation conditions. Sephadex G-15 gel-filtered corpus striatal extract was incubated at 37° for 20 min in the presence or absence of phosphorylation conditions and immediately assayed for tyrosine hydroxylase activity. 6-MPH_4 concentrations were 125 μM and 1.0 mM for phosphorylated and nonphosphorylated samples, respectively, as previously described (18). Control activities for nonphosphorylated and phosphorylated samples were 71.3 and 650 pmol of DOPA/mg of protein/min, respectively. ○, Plus MgATP and catalytic subunit; ●, nonphosphorylation conditions. Similar results were obtained in four other experiments.

TABLE 1

Characterization of corpus striatal tyrosine hydroxylase inactivation by pterins and pterin analogues

The EC_{50} values for the phosphorylated and nonphosphorylated corpus striatal tyrosine hydroxylase were determined following a 20-min preincubation with several concentrations of the specified compound at the designated pH, as described in Fig. 2. The assays were performed at the same pH as the inactivation reaction, using 25 mM PIPES buffer. The data were analyzed by computer using the algorithm described by Delean *et al.* (28, 29). The data represent the mean of three independent determinations with a standard error of less than 15%.

Compound	pH	EC_{50}		Ratio of nonphosphorylated/phosphorylated
		Nonphosphorylated	Phosphorylated	
		M		
6-MPH_4	6.0	6.9×10^{-4}	2.2×10^{-7}	3100
	7.2	1.9×10^{-3}	5.5×10^{-7}	3500
BH_4	6.0	4.2×10^{-4}	6.8×10^{-7}	610
	7.2	1.1×10^{-3}	1.4×10^{-6}	790
$DMPH_4$	6.0	8.9×10^{-4}	1.4×10^{-6}	650
2,5,6-Triamino-4-hydroxypyrimidine	6.0	5.1×10^{-4}	1.5×10^{-6}	340
6-Methyl-7,8-dihydropterin	6.0	1.0×10^{-3}	1.9×10^{-5}	53
BH_2	6.0	$>1.0 \times 10^{-3}$	1.3×10^{-4}	>7.5

Our initial hypothesis was that inactivation was mediated by fully reduced pterins. When we examined pterins that were not fully reduced, however, we also found that these compounds effected inactivation. 6-Methyl-7,8-dihydropterin, for example, inactivated tyrosine hydroxylase activity under phosphorylation conditions, with an EC_{50} value 53-fold less than that under nonphosphorylation conditions (Table 1). The EC_{50} (under phosphorylation conditions), however, was 85-fold greater than that for the fully reduced 6-MPH_4. Similarly, BH_2 was 80–100-fold less effective in mediating inactivation of phosphorylated enzyme, when compared with BH_4; however, it also showed preferential inactivation of phosphorylated tyrosine hydroxylase.

These initial studies were performed with homogenates of rat corpus striatum (17). With the availability of tyrosine hydroxylase purified from a rat transplantable pheochromocytoma (20), we characterized its inactivation by the pterin compounds. To summarize our results, we found that there were minor differences in the properties of inactivation by pterins of the purified rat pheochromocytoma enzyme, when compared with the crude rat corpus striatal preparation. We found that the extent of activation of the pure enzyme under phosphorylation conditions was 3–5-fold (20) and it was 10–12-fold with the striatal enzyme (Fig. 1). The EC_{50} values were within a factor of 2 in these systems. The stoichiometry of phosphate incorporation into tyrosine hydroxylase and the identity of the serine phosphorylated, determined as previously described (20), were not influenced by BH_4 treatment. These results suggest that the inactivation phenomenon is inherent to the enzyme itself, and the remainder of the experiments reported in the present paper were performed with the purified enzyme.

Attenuation of BH_4-mediated inactivation by tyrosine and its analogues. We were initially puzzled by the observation that BH_4 promoted the inactivation of tyrosine hydroxylase under phosphorylation conditions, because this compound is one of the substrates of the enzyme and is necessary for catalytic activity. When we tested for inactivation in the presence of tyrosine, however, we found that this substrate attenuated inactivation in a concentration-dependent fashion (Table 2). A portion of the protection afforded by tyrosine might be related to BH_4 depletion during catalysis. This result, however, was also observed when 1 mM dithiothreitol was included to regenerate BH_4. We also observed that 10 μM 3-iodotyrosine provided complete protection against inactivation by BH_4. This compound is a competitive inhibitor with respect to tyrosine (30) and will not result in depletion of BH_4 by catalysis. α-Methyl-*p*-tyrosine, also a competitive inhibitor with respect to tyrosine, diminished the degree of inactivation, but not to the extent observed with the other two compounds (Table 2).

Protection by these three substances against inactivation by BH_4 was specific, in that 200 μM concentrations of many analogues had no effect. D-Tyrosine, for example, was impotent (Table 2). This points to the requirement for the interaction of the compound with the enzyme and not to scavenging by the phenolic aromatic group or other chemical quenching mechanisms. The importance of the carboxyl group as a determinant is underscored by the failure of 200 μM tyrosinol, tyramine, tyrosinamide, and tyrosine hydrazide to attenuate inactivation. The *para*-hydroxyl group is also essential, because 200 μM

TABLE 2

Protection against inactivation of purified and phosphorylated rat pheochromocytoma tyrosine hydroxylase by BH_4: tyrosine analogue specificity

The phosphorylation of purified tyrosine hydroxylase by cyclic AMP-dependent protein kinase was performed as described in Materials and Methods. The concentration of tyrosine hydroxylase was 1.0 mg/ml (16.7 μM) and that of the catalytic subunit of protein kinase was 40 μg/ml (1 μM). Gel filtration and enzyme concentration by pressure dialysis were performed as noted in Materials and Methods. The inactivation of tyrosine hydroxylase (30 μg/ml) was performed in a final volume of 50 μl at 37° in 30 mM potassium phosphate (pH 6.0), 50 mM NaF, for 20 min. Portions (10 μl) of the compounds listed were added, before the addition of 10 μl of BH_4, to give the indicated concentration during the 20-min incubation. A 40 mM stock solution of BH_4 in 5 mM HCl was diluted in water immediately before it was added to the enzyme solution, giving a final BH_4 concentration of 1 μM. Following the 20-min incubation, the solution was gel filtered and then assayed at pH 6.0 with 125 μM 6-MPH_4, as noted in Materials and Methods, or stored at −70° before assay. The calculated specific activity assumes that the ratio of tyrosine hydroxylase and protein kinase was unchanged following gel filtration. The data represent the mean ± standard error of triplicate determinations.

Addition	Tyrosine hydroxylase activity
	nmol/min/mg of protein
None	34 ± 4
L-Tyrosine, 200 μM	98 ± 8
L-Tyrosine, 100 μM	76 ± 7
L-Tyrosine, 50 μM	54 ± 4
L-Tyrosine, 200 μM; dithiothreitol, 1 mM	91 ± 7
3-Iodo-L-tyrosine, 10 μM	110 ± 3
α-Methyl-L-*p*-tyrosine, 200 μM	77 ± 4
D-Tyrosine, 200 μM	30 ± 3
Control (no BH_4)	104 ± 6

TABLE 3

Protection against inactivation of purified and phosphorylated rat pheochromocytoma tyrosine hydroxylase by BH_4: catecholamine analogue specificity

The incubations with BH_4 were performed as described in Table 2, in the presence of the specified compound. The data represent the mean ± standard error of triplicate determinations.

Addition	Tyrosine hydroxylase activity
	nmol/min/mg of protein
None	33 ± 5
DOPA, 100 μM	28 ± 6
Dopamine, 100 μM	97 ± 11
Norepinephrine, 100 μM	91 ± 4
Epinephrine, 100 μM	107 ± 8
Isoproterenol, 100 μM	92 ± 4
Metaproterenol, 100 μM	33 ± 5
N-Methyldopamine, 100 μM	74 ± 6
3,4-Dihydroxynorephedrine, 100 μM	63 ± 8
Control (no BH_4)	114 ± 7

ortho- and *meta*-tyrosine and *O*-methyl-L-tyrosine were ineffective in nullifying BH_4 inhibition (not shown).

Attenuation of BH_4 inactivation by dopamine and its analogues. Feedback inhibitors, including 100 μM dopamine, norepinephrine, and epinephrine, prevented inactivation by BH_4 (Table 3). L-DOPA (the product of the tyrosine hydroxylase reaction), however, failed to nullify the inhibitory effect of BH_4. Analogues of dopamine had appreciable attenuating activity. Catechol derivatives (*N*-methyldopamine and 3,4-dihydroxynorephedrine) were effective (Table 3), but compounds containing one phenolic hydroxyl group (DL-octopamine and DL-synephrine) were ineffective (not shown). Isoproterenol, with 3,4-dihydroxy substituents, was very effective in attenuating the inactivation process. Metaproterenol, an isoproterenol analogue that differs only in containing 3,5-dihydroxy groups, was completely without effect (Table 3). Catecholamine metabolites without the amino group or with modified aromatic hydroxyl groups, including 100 μM homovanillic acid, vanillylmandelic acid, 3,4-dihydroxyphenylacetate, or 3-hydroxy-4-methoxyphenylethylamine, lacked a protective effect (not shown).

We also tested other aromatic compounds that might help in understanding the mechanism of inactivation. In the case of *Escherichia coli* glutamine synthetase, for example, Levine (31, 32) reported that this enzyme is inactivated by ascorbate, iron, and oxygen. This inactivation is attenuated by histidine and cysteine. In our case, however, histidine (2 mM) failed to affect the inactivation process (not shown). Not only did cysteine (2 mM) fail to attenuate the inhibition process, as a thiol reagent it promoted inactivation (not shown), as was observed with dithiothreitol (20).

Failure of free radical scavengers to attenuate BH_4-mediated inactivation of tyrosine hydroxylase. We also tested the notion that inactivation by BH_4 might be due to the generation of free radicals, with subsequent inactivation of the enzyme. Mannitol (250 mM), butylated hydroxytoluene (1 mM), ethanol (30 mM), and vitamin E (α-tocopherol acetate) (1 μM) all failed to alter the extent of inactivation by BH_4 (not shown). We also examined compounds that function as hydroxyl free radical scavengers (33). Dimethylsulfoxide (30 mM), *t*-butanol (32 mM), benzoate (30 mM), and *p*-aminobenzoate (10 mM) were ineffective in attenuating the inactivation process (not shown).

Absence of a role for reactive oxygen in inactivation of tyrosine hydroxylase by BH_4 and glutathione. Compounds such as BH_4 have the potential to react with dioxygen to form H_2O_2 and BH_2. This process is enhanced by transition metals such as iron, and tyrosine hydroxylase is an iron-containing enzyme (34, 35). BH_4 might also form other reactive substances that might inactivate the enzyme. We found that neither catalase nor peroxidase attenuated the inactivation produced by BH_4. We previously reported that these scavenger enzymes had no protective effect on the ascorbate-mediated inactivation process (18). Superoxide dismutase also failed to nullify BH_4-mediated inactivation (Table 4).

To directly test for the possible role of oxygen in inactivation, experiments were performed under near oxygen-free conditions in a nitrogen atmosphere in an anaerobic laboratory. The solutions were equilibrated and the experiments were performed in a nitrogen atmosphere that contained 100 ppm oxygen, as measured with an oximeter. The solubility of 100% dioxygen (10^6 ppm) at 1 atmosphere in pure water at 37° is 1.07 mM (36). Using Dalton's law of partial pressures, the concentration of oxygen in pure water in equilibrium with 100 ppm of

TABLE 4

Lack of effect of scavenger enzymes on BH_4 inactivation of purified and phosphorylated rat pheochromocytoma tyrosine hydroxylase

Tyrosine hydroxylase was phosphorylated and inactivation by BH_4 was performed as described in Table 2. The samples were assayed without the second gel filtration; pilot experiments indicated that these enzymes had no deleterious effect on tyrosine hydroxylase activity determinations. The data represent the mean ± standard error of triplicate determinations.

Addition	Tyrosine hydroxylase activity
	nmol/min/mg of protein
None	43 ± 5
Superoxide dismutase, 10^3 units/ml	46 ± 8
Catalase, 10^4 units/ml	40 ± 7
Peroxidase, 10^3 units/ml	41 ± 5
Control (no BH_4)	133 ± 9

the gas at atmospheric pressure is 0.107 μM. This represents an upper limit, because salts decrease the solubility of oxygen in water by a factor of 2 (36). The concentration of tyrosine hydroxylase during the inactivation reaction was 1.0 μM (60 μg of protein/ml). The oxygen, therefore, could account for inactivation of only 5–10% of the enzyme, provided that it acted stoichiometrically. The calculated oxygen content of water in equilibrium with atmospheric oxygen (21% or 2.1×10^5 ppm) is 225 μM. The extent of inactivation by BH_4 and glutathione was the same under aerobic or anaerobic conditions (Table 5).

Discussion

Following the phosphorylation of tyrosine hydroxylase by cyclic AMP-dependent protein kinase, tyrosine hydroxylase becomes less stable. This is manifested by an increased loss of activity at increased temperature (37) and the propensity to undergo apparently irreversible inactivation mediated by BH_4, 6-MPH_4, and their dihydro derivatives (Table 1) and by ascorbate and other reducing agents, such as glutathione and dithiothreitol (18). Although Kuhn and Lovenberg (27) did not determine the EC_{50} values for BH_4, 6-MPH_4, and BH_2, they used concentrations from 0.5 to 1.0 mM to effect inactivation of enzyme purified from PC12 cells (a rat-derived cell line). These correspond favorably to the EC_{50} values that we obtained with the unphosphorylated enzyme (Table 1). Inactivation of phosphorylated tyrosine hydroxylase, however, was more sensitive by 3 orders of magnitude, thereby emphasizing the importance of the phosphorylation state of the enzyme.

The irreversible nature of this process is shown by our inability to reactivate the purified or unpurified enzyme by dialysis, gel filtration, and incubation at 0° or 30° in the presence or absence of divalent metals, which might be necessary for phosphoprotein phosphatase activity (18). We considered the possibility that this inhibition might be related to proteolysis. In experiments performed in parallel with those reported for ascorbate inactivation (18), proteolysis of the enzyme purified from rat pheochromocytoma was not observed under conditions where BH_4 produced greater than a 99% loss of activity (data not shown). The data described in this paper with tetrahydropterins and presented previously with ascorbate (18) were obtained at pH 6.0. BH_4, 6-MPH_4 (Table 1), and ascorbate (not shown) also inhibited phosphorylated tyrosine hydroxylase at pH 7.2. The EC_{50} values were 2–3-fold greater at pH 7.2. Protection against BH_4 inactivation by tyrosine and dopamine was also observed at pH 7.2 (not shown).

We considered the possibility that BH_4 might react with oxygen and form H_2O_2 or other peroxides, which might be the inhibitory agents. As was the case for ascorbate (18), however, neither catalase nor peroxidase affected inactivation by BH_4. Superoxide dismutase also failed to alter the inactivation process. In solutions that were equilibrated in an atmosphere containing 100 ppm oxygen, inactivation by BH_4 and glutathione was unaltered. The calculated concentration of dissolved oxygen was about 0.1 μM and the concentration of tyrosine hydroxylase was 1 μM. These experiments rule unlikely the possibility that BH_4 forms or promotes formation of an oxygen-containing free radical.

The concentration of BH_4 that inactivated phosphorylated tyrosine hydroxylase was lower than the estimated physiological concentration of 2–12 μM (13, 14). This raises the question of the mechanism for maintaining enzyme activity in the physiological situation. This is explained, in large part, by the stabilizing effect of the tyrosine substrate, as shown in our present experiments. Physiological concentrations in the 150 μM range (38) were found to protect the enzyme against inactivation (Table 2).

We also found that the end-products of the pathway for catecholamine biosynthesis (dopamine, norepinephrine, and epinephrine) prevented the inactivation. The concentrations required to product protection seem to be higher than those expected in the cytosol (1–2 μM catecholamine), where tyrosine hydroxylase is found (39), but are consistent with the K_i of each compound (17). The product of the enzyme-catalyzed reaction, L-DOPA, failed to protect the enzyme against the inactivation process. The K_i of DOPA for unphosphorylated bovine adrenal tyrosine hydroxylase with 6-MPH_4 as reductant is about 180 μM (30) and that of dopamine for the unphosphorylated corpus striatal enzyme is about 10 μM (17). This may explain the greater effect of 100 μM dopamine, when compared with 100 μM DOPA, in providing protection against inactivation.

Based on these and previous results with ascorbate (18), we postulate that tyrosine hydroxylase, phosphorylated by cAMP-dependent protein kinase, is more labile than the nonphosphorylated form. We propose that pterins promote a conformational change in tyrosine hydroxylase, which is associated with an irreversible loss in activity. This occurs much more readily in phosphorylated tyrosine hydroxylase.

We also raise the issue of the possible physiological relevance of our findings. Because the enzyme is sensitive to subphysiological concentrations of BH_4, it might undergo inactivation in the physiological situation under nonturnover conditions in the absence or presence of low concentrations of tyrosine. The inactivation by BH_4, in principle, represents a mechanism to reduce the activity of tyrosine hydroxylase in systems activated by cyclic AMP-dependent protein kinase. On the other hand, the finding that tyrosine prevents inactivation of the phosphorylated enzyme by reducing co-substrate provides an expla-

TABLE 5

Comparison of inactivation of phosphorylated tyrosine hydroxylase under aerobic and anaerobic conditions

Phosphorylated tyrosine hydroxylase was prepared as described in Materials and Methods. The solution containing tyrosine hydroxylase and all other solutions were equilibrated in a nitrogen atmosphere for 40 min. Non-protein-containing solutions were purged with nitrogen (<5 ppm O_2) and the tyrosine hydroxylase-containing samples were gently purged (to obviate denaturation) intermittently during this period. The inactivation reaction was performed for 20 min at 37°. The concentration of tyrosine hydroxylase was 120 μg/ml (2 μM); the concentration of BH_4 was 5 μM and that of glutathione was 2 mM. After transfer to ambient atmosphere, the samples were frozen in dry ice within 1 min and stored for 48 hr before activity determinations. The samples were thawed on ice and vortexed intermittently for 5 min to permit equilibration with ambient oxygen. Inactivation under aerobic conditions was performed with the same stock solutions, except that the solutions were not purged with nitrogen. Addition of either agent to enzyme at 37° for 1 min, followed by freezing, storage, and vortexing, produced less than 20% inhibition of enzyme activity. Samples (10 μl) were added to 10 μl of assay solution for activity measurements, which were performed as previously described (22).

Addition	Tyrosine hydroxylase activity	
	Anaerobic preincubation	Aerobic preincubation
	nmol/min/mg of protein	
None	93 ± 8	97 ± 6
BH_4, 5 μM	22 ± 3	20 ± 4
Glutathione, 2 mM	56 ± 5	52 ± 5

nation for maintenance of activity in the physiological situation.

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