The Effect of Reduced and Oxidized Pteridine on Dopamine-β-Hydroxylase Activity

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SUMMARY

Tetrahydropteridine ($DMPH_4$) reacts with dopamine-β-hydroxylase anaerobically and forms a reduced enzyme intermediate which aerobically catalyzes the β-hydroxylation of tyramine-^3H. Under aerobic conditions $DMPH_4$, in combination with NADH, is effective in the stimulation of enzymic β-hydroxylation. Oxidized $DMPH_4$ inhibits the enzymic activity.

Tyrosine hydroxylase catalyzes the initial step and dopamine-β-hydroxylase the terminal step in norepinephrine biosynthesis. The two enzymes have different cofactor requirements, tetrahydropteridine ($DMPH_4$) serves as a cofactor in the enzymic tyrosine hydroxylation (1) and ascorbate in the enzymic β-hydroxylation (2). Dopamine-β-hydroxylase is a copper enzyme, and it was shown that the copper undergoes reduction and oxidation during the enzymic β-hydroxylation reaction (3–5). Ascorbate reduces the cupric copper of the enzyme (4, 5), and the reduced enzyme intermediate catalyzes the aerobic hydroxylation of the substrate (5). Other reducing agents were found to have little or no effect on the enzymic β-hydroxylation (2). $DMPH_4$ was found to be approximately 5–10% as active as ascorbate, and NADH and NADPH have shown almost no activity in the stimulation of the enzymic activity (2). More recently it was shown that the cupric copper of the enzyme can also be reduced by cysteine and the reduced enzyme intermediate catalyzes the enzymic β-hydroxylation without addition of ascorbate (6).

The present communication deals with the effect of reduced and oxidized $DMPH_4$ on dopamine-β-hydroxylase activity. We have shown that $DMPH_4$ stimulates the enzymic β-hydroxylation while oxidized $DMPH_4$ inhibits the enzymic reaction.

Methods. Dopamine-β-hydroxylase was prepared as previously described (2). The enzyme was purified to the second DEAE column eluate stage as previously described in Procedure A (3). When dopamine was used as a substrate the enzyme activity was assayed fluorimetrically (7). When tyramine-^3H was used as the substrate, the formation of the β-hydroxylated product octopamine was determined by the periodate method (8). Oxidized $DMPH_4$ was prepared by passage through oxygen for 30 minutes into a solution which contained 2 μmoles of $DMPH_4$ per 0.1 ml in phosphate buffer pH 6.5. The cuprous and cupric copper of the enzyme were determined by magnetic resonance spectroscopy (4). 2-Amino-4-hydroxy-6,7-dimethyltetrahydropteridine ($DMPH_4$) was obtained commercially.

Anaerobic experiments. The results presented in Table 1 show that when dopa-

mine-β-hydroxylase reacts anaerobically with $DMPH_4$ an enzyme intermediate is formed which is capable of aerobic β-hydroxylation of tyramine-^3H. When the enzyme reacts under the same conditions with ascorbate, the activity is higher than with $DMPH_4$. After the addition of cysteine to the hydroxylating enzyme the activity is slightly lower than after the addition of $DMPH_4$.

TABLE 1

Dopamine-β-hydroxylase activity after anaerobic reduction of the enzyme

In a Thunberg tube oxygen free nitrogen was bubbled through a mixture which contained enzyme (0.06 mg of protein), 500 units of catalase (Sigma), and 100 μmoles of acetate buffer pH 5.5 in a total volume of 0.5 ml. The tube was immersed in ice and after 10 min 0.02 μmole of the reducing agent was tipped in from the side arm of the tube. The tube was flushed with nitrogen for another 2 min and the incubation was started aerobically at 25°C after the addition of 10 μmoles of fumarate and 0.7 mμmole of tyramine-^3H (500,000 cpm). The mixture was incubated for 10 min. The results are averages from 3 experiments ± the standard error of the mean.

Reducing agent	Octopamine-^3H formed (mμmoles/mg protein)
None	0.3 ± 0.03
Ascorbate	4.6 ± 0.50
Cysteine	2.1 ± 0.20
$DMPH_4$	2.5 ± 0.30

Aerobic experiments. A loss of enzymic activity was observed when $DMPH_4$ was preincubated with dopamine-β-hydroxylase aerobically in absence of catalase. However, in presence of catalase the enzyme is protected from inactivation by $DMPH_4$. This observation provided the first clue that $DMPH_4$, like ascorbate, when added to the hydroxylating enzyme produces peroxides which inactivate the enzyme (9). The results presented in Table 2 show the effects of $DMPH_4$ and of $DMPH_4$ in combination with NADH on dopamine-β-hydroxylase activity. When ascorbate is replaced by $DMPH_4$ in an incubation mixture under aerobic conditions the enzyme shows very little activity. However, when ascorbate is replaced by $DMPH_4$ and NADH the enzyme is approximately 50% as active as in presence of ascorbate. NADH alone has almost no effect on the enzymic activity. The time course of norepinephrine formation was compared in incubation mixtures with $DMPH_4$ and NADH and in incubation mixtures with ascorbate. It was found that the initial velocity is the same in both incubation mixtures; however, the velocity increases with time in the incubation mixture with ascorbate.

TABLE 2

The effect of $DMPH_4$ and of $DMPH_4$ plus NADH on dopamine-β-hydroxylase activity

The enzyme activity was measured in a mixture containing the following components: enzyme (0.06 mg protein), phosphate buffer pH 6.0, 100 μmoles; catalase 500 units (Sigma), fumarate, 10 μmoles; dopamine-HCl, 4 μmoles, and reducing agent as indicated. The reaction mixture was incubated at 25°C for 10 min.

Reducing agent added	Relative activity
Ascorbate, 1 μmole	100
$DMPH_4$, 1 μmole	5–10
NADH, 1 μmole	0–5
$DMPH_4$, 1 μmole + NADH, 1 μmole	40–50
$DMPH_4$, 2 μmoles + NADH, 2 μmoles	50–60

Inhibition of dopamine-β-hydroxylase activity by oxidized $DMPH_4$. The results presented in Table 3 show that preincubation of the enzyme with oxidized $DMPH_4$ results in an inhibition of the enzymic activity. The inhibition by the oxidized $DMPH_4$ can be partially reversed by addition of Fe^{2+} or Co^{2+} ions to the incubation mixture.

Oxidation-reduction state of copper. A quantitative analysis performed on an enzyme sample by double integrating the magnetic resonance derivative spectrum reveals that the divalent copper of the enzyme is approximately 60% reduced after the addition of 1 μmole of $DMPH_4$ (M. Goldstein, T. H. Joh, W. E. Blumberg, and J. Peisach, unpublished observation). After the addition of ascorbate the divalent cop-

per of the enzyme is approximately reduced to the same extent (4).

Discussion. The finding that $DMPH_4$ functions like ascorbate in the enzymic β-hydroxylation reaction might be of physiological importance. In scorbutic guinea pigs the formation of norepinephrine is not decreased (10), and it is therefore possible that ascorbate is not the only compound that reduces dopamine-β-hydroxylase *in vivo*. If dopamine-β-hydroxylase is reduced *in vivo* by $DMPH_4$ then pteridines are involved in the regulation of the norepinephrine biosynthesis at the initial and at the terminal step. $DMPH_4$ in combination with NADH exhibits the maximal activity in the enzymic hydroxylation reaction at a concentration of 2×10^{-3} M, and at this concentration it is approximately half as active as ascorbate. The limited activity of $DMPH_4$ is most likely due to the inhibition of dopamine-β-hydroxylase by oxidized $DMPH_4$ which is formed during the incubation. The partial restoration of activity upon addition of Fe^{2+} or Co^{2+} ions suggest that the enzymic inhibition by oxidized $DMPH_4$ is due to its chelation with the copper of the enzyme. The high affinity of folic acid for copper (11) and the similarity in the chelating structure of 8-hydroxyquinoline with oxidized $DMPH_4$ will also support the idea that oxidized pteridine chelates with the copper of dopamine-β-hydroxylase.

TABLE 3

The inhibition of dopamine-β-hydroxylase by oxidized $DMPH_4$ and reconstitution of activity by addition of metal ions

The enzyme activity was measured in a mixture containing the same components as indicated in Table 2 except that 6 μmoles of ascorbate was added to the mixture. The oxidized $DMPH_4$ and the metal ions were added prior to the addition of ascorbate.

Additions	Norepinephrine formed (μmoles)	Activity (%)
None	2.20	100
Oxidized $DMPH_4$, 2×10^{-3} M	0.40	18
Oxidized $DMPH_4$, 2×10^{-3} M + Fe^{2+}, 2×10^{-4} M	1.60	72
Oxidized $DMPH_4$, 2×10^{-3} M + Co^{2+}, 2×10^{-4} M	1.70	77
Oxidized $DMPH_4$, 2×10^{-4} M	1.65	74
Oxidized $DMPH_4$, 2×10^{-5} M	2.00	80

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