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THE METAL REQUIREMENT OF RAT TYROSINE HYDROXYLASE

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The effect of added metals on purified rat tyrosine hydroxylase which is predominantly iron-free has been determined. The presence of 10 μM ferrous ammonium sulfate results in a ten-fold increase in the activity of enzyme containing 0.1 iron atom per subunit. The enzyme activity is half-maximal at a free ferrous iron concentration of 0.15 μM. Copper, zinc, silver, and nickel are unable to replace ferrous iron. Ferric iron is inactive unless ascorbate is included to reduce it.

Tyrosine hydroxylase catalyzes the hydroxylation of tyrosine to form dihydroxyphenylalanine, the rate-limiting step in the production of catecholamine neurotransmitters (1), utilizing oxygen and a tetrahydropterin as cosubstrates. This reaction is identical to that catalyzed by the flavoprotein phenol hydroxylases, of which p-hydroxybenzoate hydroxylase is the most studied example (2). A major difference between tyrosine hydroxylase and the flavoprotein hydroxylases is the presence of iron in the former. The presence or absence of iron in tyrosine hydroxylase has been controversial over the years (1). Recently, it has been shown that purified enzyme from either rat or bovine adrenal medulla contains one or 0.7 iron atoms per subunit, respectively (3,4). However, the catalytic role of the iron and even its redox state remain unsettled. Dix et al. (3) reported that the enzyme from rat reacts only with ferrous iron-specific chelators. In contrast, Haavik et al. (4) found that bovine enzyme had an epr spectrum characteristic of catechol-chelated ferric iron. Furthermore, catechols, which bind tightly to ferric iron, inhibit both the rat and the bovine enzyme during turnover, with K_i values of about 1 μM (1, 5).

We have recently expressed rat tyrosine hydroxylase in insect tissue culture cells using a baculovirus expression system (6). Catalytically, the cloned enzyme behaves identically to the enzyme purified from rat PC12 cells. However, the cloned enzyme contains a substoichiometric amount of iron, typically 0.1 atoms per subunit. The availability of what is essentially apo-enzyme has allowed us to demonstrate directly that tyrosine hydroxylase requires ferrous iron for activity.

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EXPERIMENTAL PROCEDURES

6-Methylpterin was synthesized by the method of Storm et al. (7) and reduced to the tetrahydro level with hydrogen over Pt_2O. Catalase was from Boehringer Mannheim. [3,5-^{3}H]Tyrosine was from Amersham. Stocks of ferrous ammonium sulfate were stored at -20 °C immediately after they were made up and were not reused after being thawed. Rat tyrosine hydroxylase was purified from insect tissue culture cells (6). The purification and characterization of the cloned enzyme will be described in detail elsewhere. The specific activity of enzyme used in this study was between 700 and 1000 nmoles dihydroxyphenylalanine produced per minute per milligram protein. The concentration of tyrosine hydroxylase was determined using an $A^{1\%}_{280}$ value of 10.4 (4). The standard conditions for the assay were 50 mM MES, 500 μM 6-methyltetrahydropterin, 100 μM [3,5-^{3}H]tyrosine (approximately 1.5 $\mu C_i/\mu mole$), 240 μM oxygen, 14 mM ß-mercaptoethanol, 75 μg/ml catalase, and 10 μM ferrous ammonium sulfate, at pH 6.4, 32 °C. Assays were started by adding either enzyme or 6-methyltetrahydropterin to the remaining assay components equilibrated at 32 °C. Most commonly, the tetrahydropterin was added within two minutes of adding enzyme to the reaction mix. Assays were quenched after two minutes by the addition of glacial acetic acid to 5%; tritiated water was separated on a Dowex 50 column. Recovery of tritiated water under these conditions was found to be 95%. Blanks contained all assay components but enzyme. The oxidation of 6-methyltetrahydropterin was monitored at 334 nm. Absorbance spectra were determined on a Hewlett-Packard Model 8452A diode array spectrophotometer. Iron concentrations were determined with a Perkin-Elmer Model 2380 atomic absorbance spectrophotometer using a graphite furnace. Oxygen consumption was determined with a Yellow Springs Model 5300 oxygen electrode. Data from measurements of the iron concentration dependence were initially analyzed by the method of Stinson and Holbrook (8), which allows total ligand concentration to be used. The approximate K_d value form this analysis was then used to correct the total ferrous iron concentration for enzyme-bound iron, assuming one iron atom per subunit. The activity as a function of free iron concentration was then fit to $v=V[Fe]/(K_d+[Fe])$, using a program written by Dr. James Robertson of Pennsylvania State University.

RESULTS AND DISCUSSION

Rat tyrosine hydroxylase which has been purified from insect cells after expression by a baculovirus system contains a substoichiometric amount of iron (6) and has a low specific activity in the absence of added iron. However, if ferrous ammonium sulfate is included in the assays, a large increase in activity of the enzyme is seen. The availability of the iron-depleted enzyme and the ability to reactivate with ferrous iron afforded an opportunity to examine the metal ion requirements of tyrosine hydroxylase.

Before doing so it was necessary to ensure that the observed activity was a valid measure of the amount of active enzyme. Initial experiments were done at pH 6.0, the most common pH for the assay of tyrosine hydroxylase. However, in our hands, the cloned enzyme is relatively unstable at this pH, as indicated by the fact that the observed activity depended strongly on whether the assay was started with enzyme or substrate tetrahydropterin, with much higher activities resulting in the former case. At pH 6.4 and above, the observed activity was independent of the method of initiating the reaction. The presence of catalase resulted in a significant

increase in activity, as has been reported by others (9). At pH 6.0 or below, the amount of ferrous iron required for maximum activity was dependent on the amount of catalase present, although the activity at high iron concentrations was not. The effects of iron and catalase were independent above pH 6.4. This ruled out the possibility that part of the effect of added metal could be due to catalysis of the breakdown of hydrogen peroxide generated by the autoxidation of the 6-methyltetrahydropterin substrate.

The presence of ferrous iron increased the rate of autoxidation of 6-methyltetrahydropterin about 3-fold at pH 6.5 and 30 °C. The presence of catalase decreased the rate to that in the absence of iron. The addition of 14 mM ß-mercaptoethanol decreased it several-fold more. The addition of both ß-mercaptoethanol and catalase decreased the rate of loss of tetrahydropterin to about 10% in 20 min. However, if oxygen consumption were monitored instead under the same conditions, catalase decreased the rate of oxygen consumption by about twofold, while ß-mercaptoethanol gave only a 30-40% decrease. Thus, even in the presence of catalase and ß-mercaptoethanol at optimal concentrations, the concentration of oxygen decreased 50% in 7 minutes in the presence of 330 µM 6-methyltetrahydropterin. Consistent with these results, the rate of production of dihydroxyphenylalanine by the enzyme under these conditions was only linear for two minutes in the presence of ferrous iron. Because of the depletion of substrates in the presence of ferrous iron, it was necessary to keep assays to two minutes or less to obtain strict dependence upon ferrous iron. Finally, blanks containing no enzyme were unaffected by the presence of catalase, added metal, or ß-mercaptoethanol.

ß-Mercaptoethanol is typically included in assays of tyrosine hydroxylase to reduce any dihydropterin formed by autoxidation (10). Our results are consistent with this, but point out that the autoxidation also consumes oxygen which is not replaced. Over the course of an assay running 20 minutes or longer, most of the oxygen present would be depleted. The presumed role of catalase is to destroy the hydrogen peroxide formed during autoxidation. In addition, in the absence of catalase, hydrogen peroxide would be expected to react with any ferrous iron present, generating ferric iron and highly reactive hydroxyl radical in a Fenton reaction (11). The increased rate of autoxidation of tetrahydropterin in the presence of ferrous iron is consistent with the ability of ferrous iron to catalyze autoxidation reactions by overcoming the spin-forbidden nature of the reaction of triplet oxygen.

When the above complications were allowed for, it was possible to examine the metal ion requirement of tyrosine hydroxylase. No lags in the production of dihydroxyphenylalanine were detected when assays in the presence of added ferrous iron were started with enzyme, indicating that the uptake of ferrous iron by the enzyme is relatively rapid. (A lag of less than about 4 seconds would not have been detected.) The degree of activation of iron-depleted enzyme by 10 µM ferrous ammonium sulfate was consistent with the iron content of the enzyme. Thus, with four enzyme

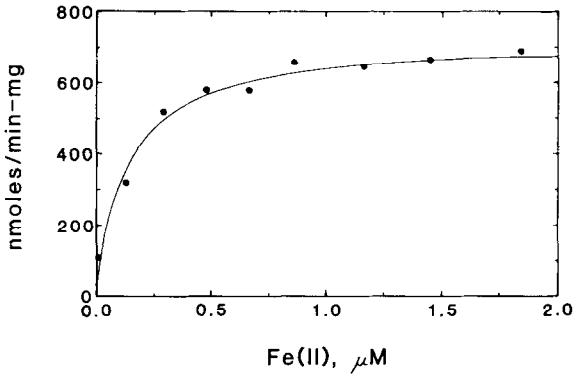

Figure 1. Dependency of the activity of tyrosine hydroxylase on the concentration of ferrous iron. The activity of rat tyrosine hydroxylase (0.19 μM) containing 0.07 iron atoms per subunit was determined in the presence of ferrous ammonium sulfate as described in Experimental Procedures. The data have been corrected for enzyme-bound iron. The line is a fit to $v=V[Fe_{free}]/(K_d+[Fe_{free}]$, with $K_d=0.15$ μM.

preparations which contained an average of 0.098 atoms of iron per subunit, an average increase in activity of 9.6-fold was seen. This is in contrast to our experience with the enzyme purified from bovine adrenal medulla, which is fully active in the absence of added iron (Fitzpatrick, P.F., unpublished observations). The presence of ferrous ammonium sulfate did not affect the K_M value for either tyrosine or 6-methyltetrahydropterin or the pH-rate profile of the rat enzyme (results not shown). These results are most consistent with an absolute requirement for ferrous iron for activity, rather than with iron acting as a non-essential activator.

The effect of the concentration of ferrous iron on the activity of tyrosine hydroxylase was determined. The activity was half-maximal at a concentration of free ferrous iron of 0.15 μM (Figure 1). No sign of cooperativity was detected. It should be noted that the limiting specific activity reached in the experiment of Figure 1 was 730 nmoles of dihydroxyphenylalanine produced per minute per milligram of enzyme. This

Table 1

Effect of Alternative Metals on the Catalytic Activity of Tyrosine Hydroxylase

metal	Activity (nmoles/min-mg)	
none	95.6	(8.2)*
10 μM $Fe(NH_4)_2(SO_4)_2$	931	(32)
50 μM $Fe_2(SO_4)_3$	101	(0.4)
100 μM CuSO4	94.9	(7)
100 μM $ZnSO_4$	8.4	(1.5)
100 μM $AgNO_3$	103	(4.9)
100 μM $NiCl_2$	64.5	(1.3)

Conditions: 100 μM tyrosine, 500 μM 6-methyltetrahydropterin, 240 μM oxygen, 75 μg/ml catalase, 14 mM ß-mercaptoethanol, 50 mM MES, pH 6.4, 32 °C. Assays were started by adding 6-methyltetrahydropterin approximately two minutes after adding tyrosine hydroxylase to the complete reaction mix containing the indicated metal.
*Standard deviation.

is about twice the specific activity of typical preparations of the rat enzyme purified from rat tissues when they are assayed at 37 °C instead of 32 °C (12). Therefore, the iron-deficient enzyme is completely reconstituted under the conditions of these experiments.

The ability of other metals to replace ferrous iron was also determined. Of all the metals tested, increased activity was only seen in the presence of ferrous iron (Table 1). However, if 1 mM ascorbate was included in the assays, ferric iron, but no other metal, was also effective (results not shown), consistent with reduction of the iron to the ferrous state. Ascorbate had no effect on the activity in the presence of ferrous iron if fresh solutions of the latter were used. It has previously been reported that tyrosine hydroxylase is much more active in the presence of ascorbate (13). However, ferrous ammonium sulfate will oxidize over the course of several hours if exposed to oxygen. Thus, if no precautions are taken a presumed stock of ferrous iron will contain significant amounts of ferric iron. The addition of ascorbate will reduce the iron back to the ferrous state, resulting in an increase in enzyme activity due to the presence of the latter.

In conclusion, these results demonstrate that tyrosine hydroxylase requires ferrous iron for activity. With iron-depleted enzyme, the increase in activity with ferrous iron is equal to the expected increase in the iron content of the enzyme. Further, no other metals can replace the ferrous iron.

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