# KINETIC PROPERTIES OF BRAIN TYROSINE HYDROXYLASE AND ITS PARTIAL PURIFICATION BY AFFINITY CHROMATOGRAPHY*

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## Summary

The tyrosine hydroxylase activity of sheep brain caudate nuclei homogenates was solubilized by treatment with 0.2% Triton X-100 and concentrated by ultrafiltration. Although the enzyme shows an absolute requirement for a reduced tetrahydropteridine ($DMPH_4$) as cofactor, concentrations > 2 mM are inhibitory. The maximal reaction velocity is stimulated two-fold by the inclusion of 0.5 mM $Fe^{2+}$ in the assay. However, the apparent Michaelis constants, $K_m$, for L-tyrosine and $DMPH_4$ are not affected by $Fe^{2+}$ and values of 0.1 and 0.33 mM were obtained for these parameters. A five-fold purification of the enzyme concentrate was achieved by affinity chromatography on iodotyrosine-substituted agarose. Active eluate fractions were distinctly turbid, suggesting that intermolecular aggregation of the tyrosine hydroxylase protein occurs as a concomitant of the purification procedure.

Tyrosine hydroxylase (EC 1.14.3.1), a monooxygenase which catalyzes the initial step in the biosynthesis of norepinephrine, has been demonstrated in the adrenal medulla, brain and sympathetically innervated tissues (1, 2). The enzyme requires a tetrahydropteridine as cofactor (1, 3, 4) and the activity of the soluble adrenal enzyme, partially purified by ammonium sulfate fractionation (5, 6), is stimulated by $Fe^{2+}$ and inhibited by a wide variety of iron chelating agents (4-7). This behavior suggests an iron requirement for the hydroxylation reaction in which tyrosine is converted to 3, 4-dihydroxyphenylalanine (DOPA).

Although tyrosine hydroxylase from bovine adrenal glands has been thoroughly

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characterized with respect to its kinetic properties (1-7), the brain enzyme has been less extensively investigated and the kinetic parameters reported for it by various authors (8-10) are somewhat discrepant. From the work of Cote and Fahn (11) and others, it is evident that a considerable enrichment of brain tyrosine hydroxylase activity may be achieved by dissection of the caudate nucleus prior to homogenization. For this reason, the caudate nuclei of sheep brains were used as the enzyme source for the studies reported here. Certain kinetic parameters of brain tyrosine hydroxylase have been determined and a protocol devised by which the crude enzyme may be partially purified, using the technique of affinity chromatography.

## Materials and Methods

[U-$^{14}$C] L-tyrosine (446 μC/μmole) was obtained from New England Nuclear Corp. and used without further purification; 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine ($DMPH_4$) was supplied by Calbiochem and NSD-1055 (p-bromo-m-hydroxybenzyloxyamine) by Smith-Nephews Ltd.

Tyrosine hydroxylase activity was measured by a modification of the method of Nagatsu et al (5). Reagent portions were scaled down to give a final incubation volume of 220 μl. In the calculations, a 65% recovery of DOPA was assumed and verified periodically with $^{14}$C-DOPA. Each assay contained 0.2 M sodium acetate, pH 6.2, 1 mM $DMPH_4$, 0.1 mM NSD-1055, 0.1 M mercaptoethanol and 45 μM L-tyrosine containing $1.6 \times 10^5$ counts/min. For those assays requiring $Fe^{2+}$, freshly prepared ferrous ammonium sulfate solution was added as well. Incubations were carried out at 37° for 15 min in a metabolic shaker. The reaction was stopped by the addition of 2 ml 5% TCA containing 20 μg of L-DOPA as carrier.

Caudate nuclei (12-14 g) were dissected from 10 sheep brains and either kept frozen at -70° or homogenized immediately in 9 volumes of ice-cold 0.25 M sucrose in an all-glass homogenizer. All subsequent steps were carried out at 0-4°. The

homogenate was centrifuged at 2000 g for 15 min, the sediment suspended in 1/2 the original volume of sucrose and recentrifuged. The supernatants were combined and the enzyme was solubilized by treatment with 0.2% Triton X-100, followed by centrifugation at 105,600 g for 1 hr. The supernatant was concentrated 10 to 15-fold by vacuum ultrafiltration and stored anaerobically at -20°. Under these conditions the activity of the concentrate was stable for at least three months.

Sepharose 4 B (Pharmacia ) was activated with cyanogen bromide according to the procedure of Cuatrecasas (12). Coupling of the inhibitor 3-iodotyrosine to the activated agarose was accomplished at pH 11 by adding 90 μmoles per ml resin and stirring the slurry for 18 hrs. at 4°. The degree of modification, estimated by a method for determining protein bound iodide (13), was 2-4 μmoles of 3-iodotyrosine bound per ml resin. Column chromatography of the enzyme concentrate was performed as noted in the legend to Fig. 3. Eluate fractions were monitored for activity and protein content, which was estimated by the Folin-Lowry procedure (14), using bovine serum albumin as standard.

## Results and Discussion

It was observed consistently that tyrosine hydroxylase activity was enhanced two to four-fold by $Fe^{2+}$ in the detergent-solubilized enzyme fractions, while little or no stimulation by $Fe^{2+}$ was observed for the homogenate or the sucrose extract. Accordingly, the Triton-treated enzyme concentrate was used to determine the apparent Michaelis constants ($K_m$) for L-tyrosine and $DMPH_4$, in the presence and absence of $Fe^{2+}$. Linweaver-Burk double-reciprocal plots of the data obtained for tyrosine are shown in Fig. 1. It is evident that only the maximal reaction velocity is affected by supplementing the assay with $Fe^{2+}$ and that the activity is enhanced by a factor of 2.2 (the ratio of the two slopes). Both plots extrapolate to approximately the same intercept on the abscissa. The apparent $K_m$ obtained for L-tyrosine is 0.1 mM in either case.

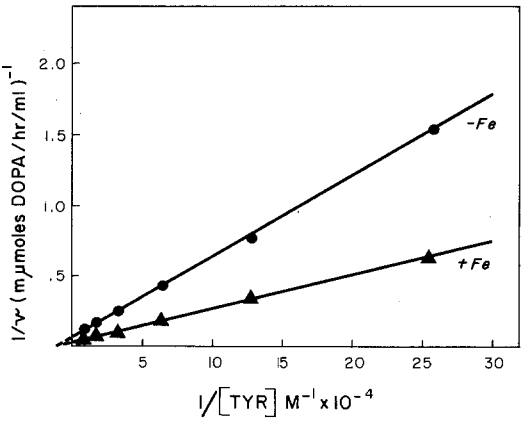


Figure 1. Linweaver-Burk plots of sheep brain tyrosine hydroxylase activity versus L-tyrosine concentration, in the absence (●) and presence (▲) of 0.5 mM $Fe^{2+}$. Incubation mixtures consisted of 80 μl of enzyme concentrate (0.25 mg), 20 μl of either water or ferrous ammonium sulfate solution and 40 μl of a buffered $DMPH_4$ solution. The reaction was initiated by the addition of 80 μl of a suitable tyrosine mixture and incubations were carried out at 37° for 15 min. Each assay was performed in duplicate with an appropriate blank heated to 80° for 10 min prior to incubation. The final concentration of $DMPH_4$ was 1 mM and the tyrosine concentration was varied from 3.9 to 125 μM.

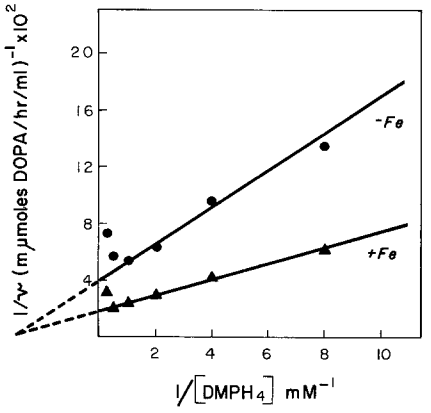


Figure 2. Linweaver-Burk plots of tyrosine hydroxylase activity versus $DMPH_4$ concentration, in the absence (●) and presence (▲) of 0.5 mM $Fe^{2+}$. Assays were performed with 1 mg of enzyme concentrate, under the conditions described in the legend to Figure 1. The final concentration of tyrosine was 44.6 μM and the $DMPH_4$ concentration was varied from 0.125 to 4 mM.

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Although the enzymatic activity showed an absolute requirement for $DMPH_4$, both in the presence and absence of $Fe^{2+}$, concentrations > 2 mM were inhibitory.

Consequently, the Linweaver-Burk plots shown in Fig. 2 are nonlinear. Nevertheless, the linear portions of each curve extrapolate to the same intercept on the abscissa and the apparent $K_m$ for $DMPH_4$ is 0.33 mM in either case. The apparent $K_m$ values of 0.1 and 0.33 mM found for L-tyrosine and $DMPH_4$ with the brain enzyme are comparable to the values of 0.1 and 0.5 mM obtained by Ikeda et al(15) for the adrenal enzyme.

A wide variety of tyrosine analogues and catechol derivatives are known to inhibit adrenal tyrosine hydroxylase in vitro (5, 6). Such findings have been used, in conjunction with in vivo studies, to formulate a model of feedback regulation of catecholamine biosynthesis in the nervous system. Accordingly, certain representative examples of such compounds were assessed for their ability to inhibit brain tyrosine hydroxylase. It was found that the metabolites dopa, dopamine and norepinephrine were moderately effective inhibitors at a concentration of 1 mM.

In recent years the technique of affinity chromatography (12) has been exploited in the purification of proteins whose biological activity is labile to more conventional chromatographic methods. A substrate-analog inhibitor is covalently attached to a suitable matrix, usually agarose or polyacrylamide, thereby obtaining a solid support capable of binding specifically and reversibly the protein of interest. The two substrate analogues, L-phenylalanine and 3-iodo-L-tyrosine were both competitive inhibitors with respect to tyrosine and from the appropriate Linweaver-Burk plots, values of 0.5 and 100 μM were obtained for their respective inhibitor constants, $K_i$. Due to its more favorable affinity, 3-iodotyrosine was used to prepare the affinity chromatography resin described in the experimental section.

The results obtained for the chromatography of the enzyme concentrate on both unmodified and 3-iodotyrosine-substituted agarose are shown in Fig. 3. It is evident that in the former case (upper curve) no retention of tyrosine hydroxylase occurred and the activity-protein elution profiles were essentially coincident. About 89% of the applied activity was recovered in this peak. In contrast, the elution

profile for the iodotyrosine-modified resin (lower curve) shows that about 2/3 of the total protein applied to the column passed through unretarded and was devoid of enzymatic activity. The adsorbed tyrosine hydroxylase protein was subsequently eluted by dilute alkali (1 mM KOH), with recovery of 52% of the applied activity. In scaled-up versions of the experiment depicted in Fig. 3, purifications of as much as five-fold, relative to the crude enzyme concentrate, have been achieved. It is evident, therefore, that sheep brain tyrosine hydroxylase may be at least partially purified by means of affinity chromatography. Hence, it should be a useful tool for the purification of this enzyme from various sources. It is noteworthy, however, that the enzymatically active fractions eluted from the iodotyrosine resin are heavily turbid, suggesting intermolecular aggregation of the tyrosine hydroxylase protein as a

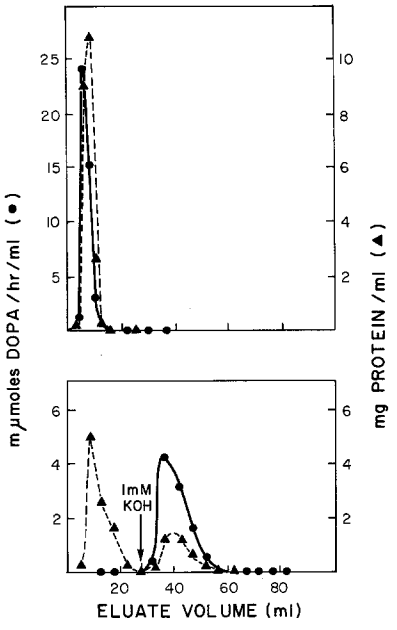


Figure 3. Affinity chromatography at 4° of brain tyrosine hydroxylase on iodotyrosine-modified (lower) and unmodified (upper) Sepharose 4B. Columns (0.7 x 12 cm) were equilibrated with 0.1 M potassium phosphate buffer, pH 6.2, and 1 ml (50 mg) of the Triton-solubilized enzyme concentrate was applied. The columns were developed with the above buffer and 4 ml fractions were collected. For the lower curve, the eluant was changed to 1 mM KOH, pH 10, at the arrow. In this case, each collecting tube contained 1 ml of 0.1 M potassium phosphate, pH 6.2, to neutralize the effluent.

concomitant of the purification procedure. Furthermore, preliminary disc gel electrophoretic data suggest that the enzyme is already aggregated in the sample applied to the column. Hence, it is not possible to prevent this behavior by manipulation of column parameters. It is remarkable, nonetheless, that such aggregates appear to be fully active and to be sterically unhindered in their ability to bind reversibly to the resin (16).

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