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Oxygen dependence of tyrosine hydroxylase

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Summary. The effects of dioxygen on tyrosine hydroxylase (TH) activity was studied, measuring the formation of DOPA from tyrosine, ^3H_2O from 3,5-^{3}H-tyrosine, or by direct oxygraphic determination of oxygen consumption. A high enzyme activity was observed during the initial 1–2 min of the reactions, followed by a decline in activity, possibly related to a turnover dependent substoichiometrical oxidation of enzyme bound Fe(II) to the inactive Fe(III) state. During the initial reaction phase, apparent K_m-values of 29–45 μM for dioxygen were determined for all human TH isoforms, i.e. 2–40 times higher than previously reported for TH isolated from animal tissues. After 8 min incubation, the K_m (O_2)-values had declined to an average of 20 ± 4 μM. Thus, TH activity may be severely limited by oxygen availability even at moderate hypoxic conditions, and the enzyme is rapidly and turnover dependent inactivated at the experimental conditions commonly employed to measure in vitro activities.

Keywords: Catecholamines – Human – Hypoxia – Oxygen – Tyrosine hydroxylase

Abbreviations: BH_4, (6*R*)-L-*erythro*-5,6,7,8-tetrahydrobiopterin; 4aOH-BH_4, 4a-hydroxytetrahydrobiopterin; q-BH_2, quinonoid dihydrobiopterin; DTT, dithiothreitol; hTH, human tyrosine hydroxylase; PAH, phenylalanine hydroxylase; TH, tyrosine hydroxylase; *Enzymes*: phenylalanine hydroxylase, EC 1.14.16.1; tyrosine hydroxylase, EC 1.14.16.2

Introduction

Tyrosine hydroxylase (TH) catalyzes the initial and rate-limiting step in the biosynthesis of the catecholamine neurotransmitters dopamine, noradrenaline and adrenaline. TH is highly homologous in terms of both protein sequence and catalytic mechanism to phenylalanine hydroxylase (PAH) and tryptophan hydroxylase (TPH). In the TH reaction, dioxygen, L-tyrosine, and tetrahydrobiopterin (BH_4) are converted to L-dihydroxyphenylalanine (L-DOPA) and 4a-hydroxytetrahydrobiopterin (4aOH-BH_4) (Eq. (1)) (Haavik and Flatmark, 1987). The 4a-hydroxytetrahydrobiopterin rapidly dehydrates either enzymatically or nonenzymatically to form the slightly more stable quinonoid dihydrobiopterin (q-BH_2) (Haavik and Flatmark, 1983). Based on steady-state kinetic studies of the recombinant rat TH catalyzed reaction, a sequential reaction mechanism has been proposed with an ordered binding of tetrahydrobiopterin, dioxygen and then tyrosine (Fitzpatrick, 1991).

$$\text{L-tyrosine} + BH_4 + O_2 \rightarrow \text{L-DOPA} + \text{4aOH-}BH_4 \quad (1)$$

All aromatic amino acid hydroxylases contain a single atom of non-heme iron per enzyme subunit. The structure of the iron site in TH has been studied by spectroscopy (Meyer-Klaucke et al., 1996) and by X-ray crystallography (Goodwill et al., 1997).

Human and animal studies have demonstrated that deficiency in any of the three TH substrates can be rate limiting under certain physiological or pathological conditions. A defective DOPA production due to congenital BH_4 deficiency (Thony and Blau, 2006) may be treated with dietary BH_4 supplementation. Accumulation of phenylalanine, as occurs in phenylketonuria, will competitively inhibit the transport of tyrosine into the brain and also directly inhibit the TH reaction. Thus, tyrosine supplementation may partially alleviate the toxic effects of phenylalanine (Koch, 1996). Several studies have demonstrated a decreased biosynthesis of catecholamines and serotonin in the nervous system of animals subjected to hypoxia (Davis, 1976; Hayashi et al., 1990). However, it is not clear whether this reduction is directly related to substrate depletion of the enzyme and how the environmental dioxygen

levels translate into in vivo oxygen concentrations in catecholaminergic cells.

In previous kinetic studies of TH from different mammalian species, a wide range of values for its oxygen affinity have been reported, i.e. K_m-values of 2–16 mmHg, corresponding to dioxygen concentrations of 3–24 μM at 25 °C and normobaric pressure (Ikeda et al., 1966; Fisher and Kaufman, 1972; Numata et al., 1977; Katz, 1980; Fitzpatrick, 1991), but higher K_m-values have been reported when synthetic analogues of BH_4 were used as electron donors in the TH reaction (Fisher and Kaufman, 1972). Thus, estimated K_m-values for O_2 vary by a factor of more than 20. Similarly, uncertainties exist regarding the tissue partial pressures of oxygen in living animals. Still, it is clear that at sea level the oxygen tensions in rat brain, kidney and liver (Feinsilver et al., 1987; Rolett et al., 2000) may approach the K_m levels suggested for rat TH and potentially be rate limiting. Smith et al. (1977) reported oxygen tension values in the cortex of rabbit brain between 7 and 30 μM during normoxic conditions and Rolett et al. (2000) found an oxygen concentration of 20 μM in rat brain. Thus, at high altitude, the partial oxygen tension in peripheral tissue of humans most probably will reach levels in the range of the K_m-values of rat TH, especially in subjects not fully acclimatized.

The effects of dioxygen on human TH (hTH) have previously not been studied, neither on the isolated enzyme nor in intact systems. hTH exists as four different isoforms (hTH1-hTH4) generated by alternative splicing of pre-mRNA (Grima et al., 1987). All isoenzymes have been synthesized in *E. coli* and characterized (Haavik et al., 1991). The present kinetic study of human TH represents the first quantitative study of the effects of dioxygen concentration on its enzymatic activity, with particular emphasis on the range of oxygen tensions that may occur in human tissues.

Using three different assay procedures, we demonstrate that the K_m-values for dioxygen for the purified TH isoforms are unexpectedly high, suggesting a direct role of tissue hypoxia in the regulation of catecholamine biosynthesis. Similarly, using intact rat pheochromocytoma (PC12 cells), we also found that the in situ TH activity decreased immediately upon reduction of the ambient dioxygen concentration.

Materials and methods

Materials

Argon (99.996%) and oxygen/nitrogen mixtures of the following defined concentrations (% volume) were purchased from Hydro Gas (Rjukan, Norway): 0.103 ± 0.002, 0.517 ± 0.01, 0.982 ± 0.02, 1.96 ± 0.04, 5.09 ± 0.10, 21.0 ± 0.42 and $100 \pm 2\%$ dioxygen. Intermediate concentrations of dioxygen were prepared by mixing these basic gases. The final solution oxygen concentrations were verified by oxymetric measurements (Fossbakk and Haavik, 2005). 6R-Tetrahydrobiopterin (BH_4) was purchased from Dr. B. Schircks laboratories, Switzerland, while all other reagents used were of analytical grade and purchased from Sigma, St. Louis, MO, USA. hTH isoforms 1, 3 and 4 (hTH1, hTH3, hTH4) were synthesized in *E. coli* and purified to homogeneity (Haavik et al., 1991). Bovine TH (bTH) was purified from the cytosolic fraction of adrenal medulla extracts (Haavik et al., 1988). The concentration of purified TH was determined by the absorbance at 280 nm as described (Haavik et al., 1988). Human phenylalanine hydroxylase (hPAH) was synthesized in *E. coli* and purified as described (Knappskog et al., 1993).

Radiochemical assay of TH activity

TH activity was measured as described by Fitzpatrick et al. (1990). Reactions were performed in 2 ml stirred glass vials with septum, containing 0.5 ml reaction mixture. The solutions were evacuated before use and flushed with the desired gas mixture for 15 min at 5, 15, 20, 25, 30, or 37 °C before addition of 3 μl TH ($0.13\,mg \cdot ml^{-1}$) through the septum ($0.78\,\mu g \cdot ml^{-1}$ final conc.). The enzyme was preincubated on ice with 10 μM Fe(II) ammonium sulfate for 30 min. In other experiments the reactions were performed in 1 ml vials containing 400 μl reaction mixture. At 20–30 sec intervals, 50 μl samples were removed and assayed for formation of ^3H_2O by liquid scintillation counting or DOPA by high-performance liquid chromatography with fluorimetric detection (Haavik and Flatmark, 1980). The assay mixture contained 200 μM BH_4, 2 mM dithiothreitol, 4–105 μM Fe(II) ammonium sulfate, $0.1–0.5\,mg \cdot ml^{-1}$ catalase, and 25 μM or 50 μM tyrosine in 40 mM Na-Hepes pH 7.0.

Oxygraphic measurements

Oxygraphic assays of TH and PAH activities were carried out in a high-resolution respirometer (Oroboros Oxygraph, Innsbruck, Austria) (Gnaiger et al., 1995) at 30 and 25 °C. For TH assays, the initial tyrosine concentrations were 25 or 50 μM, while PAH was assayed using 500 μM phenylalanine (Fossbakk and Haavik, 2005). Concentrated enzyme was preincubated for 15 min in a mixture containing the final concentrations of assay components, but without cofactor, before the reaction was initiated by injection of 3–20 μl enzyme into the reaction mixture. Initial oxygen concentrations were varied by adding pure argon into the gas phase of the partially opened oxygraph chamber. The chamber was then closed for recording chemical oxygen consumption before the reaction was initiated by addition of enzyme. The final TH subunit concentrations were 23–$52\,\mu g \cdot ml^{-1}$. In some control experiments, the reaction was started by injection of the cofactor BH_4, with the enzyme already present in the reaction chamber. The exponential time constant of the polarographic oxygen sensors in different chambers ranged between 3.2 and 4.4 sec, and was used for deconvolution of the recorded data (Gnaiger et al., 1995). Dioxygen concentration was digitally recorded at time intervals of 1 or 2 sec. Reaction rates were calculated as the negative time derivatives of dioxygen concentration and corrected for the linear oxygen dependence of instrumental background. Enzyme catalyzed reaction rates, v_{O_2} ($nmol \cdot min^{-1} \cdot ml^{-1}$), were further corrected for chemical background owing to autoxidation of the incubation medium containing substrates and cofactors without enzyme. The typical background oxidation of the incubation medium was approximately $4.52 \pm 0.258\,nmol \cdot min^{-1} \cdot ml^{-1}$ when the oxygen concentration was 240 μM, where autoxidation of BH_4 could account for $3.52 \pm 0.43\,nmol \cdot min^{-1} \cdot ml^{-1}$, DTT $0.62 \pm 0.11\,nmol \cdot min^{-1} \cdot ml^{-1}$, and free Fe(II) for $0.41 \pm 0.18\,nmol \cdot min^{-1} \cdot ml^{-1}$ (all data are presented as means ± SEM, $n = 4$). By reducing the initial concentration of Fe(II) from 100 μM used in conventional TH assays to 4 μM, its background activity was reduced ten-fold (Fossbakk and Haavik, 2005).

Tyrosine did not contribute significantly to the background oxidation, but seems to have a stabilizing effect and lower the autoxidation of DTT. Background corrections of reaction rates were performed at each oxygen concentration over the entire experimental oxygen range. Chemical background oxygen consumption was also a linear function of dioxygen concentration, with the intercept not significantly different from zero. The linear regression, therefore, was forced through zero, yielding a slope of 10.8 pmol · min^{-1} · ml^{-1}/μM O_2 that was used in the calculations.

Analysis of oxygraphic enzyme kinetics

The derivative of the O_2 consumption curve obtained from the oxygraph was used to obtain values for enzymatic activities at different times after addition of enzyme. Background consumption of O_2 was subtracted before analysis was performed. The enzyme activity was measured at different oxygen concentrations. A maximum rate was observed immediately after addition of the enzyme, followed by a rapid decline of enzyme activity to a lower but constant level. For calculations of K_m and k_{cat} at different times, the enzyme activity at the given time was plotted against initial O_2 concentration and the data was fitted by a hyperbolic Michaelis-Menten equation. For accurate determination of the initial rate, we used the experimental data including the data point with maximal activity and the following ones up to 3 min after addition of enzyme. These data points were used to obtain the activity at the time of enzyme addition by fitting to an exponential decay equation (Eq. (2)), where V_t is the observed rate at time t, V_{HA} reaction rate in the 'high-activity' state, V_{LA} the reaction rate in the 'low-activity' state and k the decay constant.

$$V_t = (V_{HA} - V_{LA}) \exp(-kt) + V_{LA} \quad (2)$$

For all enzymes, aliquots of 10 μl were removed at selected time intervals, mixed with 40 μl ice cold ethanol containing 1 M acetic acid, centrifuged for 10 min and the supernatant was injected into an Agilent 1100 HPLC chromatographic system with fluorimetric detection for determination of tyrosine, 5-OH-tryptophan, and L-DOPA (Haavik and Flatmark, 1980). In some experiments, an intrinsic standard was added to the reaction mixture (1.0 μM 5-OH-tryptophan in the PAH assay) to increase the precision of the HPLC analysis (Fossbakk and Haavik, 2005).

NADH coupled assay of TH activity

The effect of iron chelators on the activity of hTH was determined using a coupled assay with dihydropteridine reductase. The assay mixture contained 200 μM BH_4, 0.45 units · ml^{-1} sheep dihydropteridine reductase, 190 μM NADH, 4 μM Fe(II) ammonium sulfate, 0.1 mg · ml^{-1} catalase, and 50 μM tyrosine in 40 mM Na-Hepes pH 7.0. The assay mixture was allowed to equilibrate at 25 °C before hTH3 was added to a final concentration of 12.4 μg · ml^{-1}. The absorbance was continually monitored at 340 nm using a Cary 50 Bio spectrophotometer. Fe(II) or Fe(III) specific chelators were added to the reaction mixture. Water replaced the chelator in the control assays. The decrease in absorption in assays lacking TH was subtracted to correct for autoxidation of Fe(II) and BH_4.

Cell culture

Rat pheochromocytoma cells (PC12 cells) were grown in 75 cm^2 flasks (40 ml medium) and on cell culture dishes (100 × 22 mm) in Dulbecco's Modified Eagle's Medium (DMEM) (Cambrex) containing 10% inactivated horse serum, 5% fetal bovine serum, penicillin (100 U · ml^{-1}) and streptomycin (100 μg · ml^{-1}) in an atmosphere of 5% CO_2 at 37 °C. The culture medium was changed three times a week. Experiments were performed on cells grown to confluence on culture dishes and washed in a medium consisting of 150 mM NaCl, 5 mM KCl, 25 mM Hepes (pH 7.4), 2.2 mM $CaCl_2$, 0.8 mM $MgCl_2$ and 5 mM glucose. The cells were then incubated with 10 μCi 3,5-^{3}H-tyrosine for 1 h in an atmosphere of 5% CO_2 at 37 °C. After incubation, extracellular ^{3}H-tyrosine was removed. Cells from two dishes were resuspended in 2.5 ml medium and transferred to an oxygraph chamber. The oxygen concentration in the oxygraph chamber was varied by adding argon to the open chamber. Experiments were performed in oxygen concentrations in the range of 20–200 μM dioxygen. Aliquots of 50 and 200 μl were removed and assayed for formation of ^3H_2O by liquid scintillation counting.

Results

Conventional TH activity assays

In Fig. 1A, representative experiments testing the effect of different concentrations of dioxygen on the hTH1 activity (rate of ^3H_2O formation) are shown. The enzyme activity was not linear with time, as the reaction rates were highest initially, but were relatively linear between 1 and 8 min incubation. The deviation from linearity may indicate

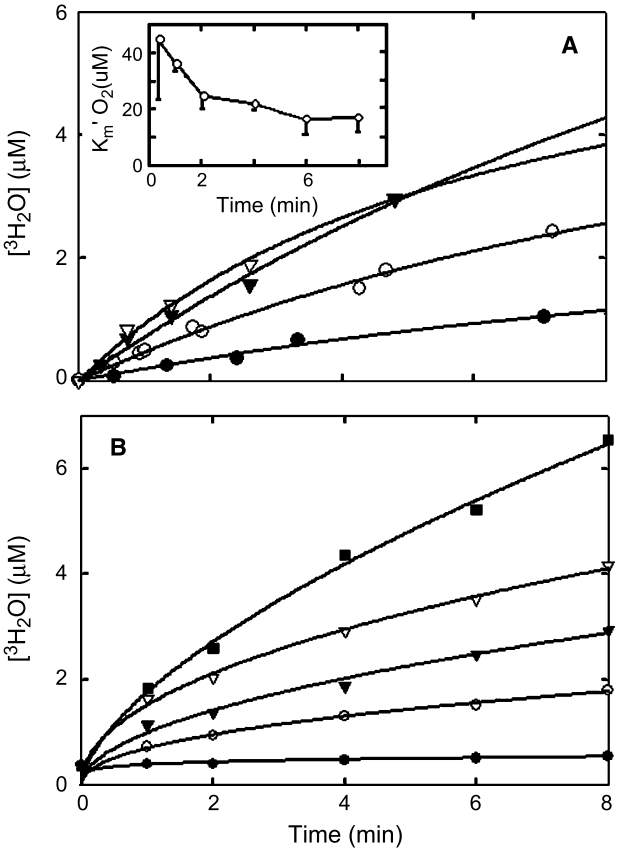

Fig. 1. Radiochemical assay of hTH1 activity. (**A**) The generation of ^3H_2O was measured in solutions equilibrated with different concentrations of dioxygen; 7.8 (●), 30.2 (○), 240 (▼), and 1142 μM (▽) (see Experimental procedures). The concentration of iron(II) was 4 μM. The kinetic data in Fig. 1A were fitted to a hyperbolic curve and the apparent K_m-values were calculated at different incubation times (inserted figure). (**B**) The TH reaction was performed as in Fig. 1A, but the concentration of free Fe(II) was 105 μM. The generation of ^3H_2O was measured in solutions equilibrated with different concentrations of dioxygen; 0 (●), 5.3 (○), 13.3 (▼), 20 (▽) and 60 μM (■)

either a partial inactivation of the enzyme during the reaction, a kinetic burst, or substrate depletion. The latter possibility was ruled out, as less than 5% of the tyrosine was consumed after 5 min incubation, and direct measurements (see "oxygraphic assay" below) confirmed that the concentrations of dioxygen and BH_4 were unaltered during the assay. The K_d-values for the iron complexes of human TH isoforms have been determined experimentally to be 0.9–3 μM at pH 6.5 and <1 μM at neutral pH (Haavik et al., 1992). Thus, the possibility was considered that the enzyme could become unsaturated with Fe(II) during the reaction. However, when the kinetic experiments were repeated using a concentration of free Fe(II) that was 26-fold higher (105 μM, Fig. 1B) the initial reaction burst was comparable to that observed using either 4 or 10 μM Fe(II), and the estimated K_m-values were similar (see below).

The TH activity as a function of initial oxygen concentrations shown in Fig. 1A and B could be fitted to a hyperbolic (Michaelis-Menten) curve. However, the apparent K_m-values for dioxygen were highest after short incubation periods (36.1 ± 2.7 μM at 1 min), but leveled of at 16.2 ± 4.6 μM after 6–8 min incubation (Fig. 1A) (mean ± SEM, $n = 10$). The lack of a stable K_m-value during incubation is consistent with a deviance from a mono-phasic steady state reaction.

Oxymetric assay of TH activity

In the experiment shown in Fig. 1 and in previous studies on the oxygen dependency of TH, the enzyme activity has been determined using assays where aliquots of the reaction mixture were removed and analyzed for the formation of either L-DOPA or ^3H_2O, while the oxygen tension has not been measured directly during the reaction (Ikeda et al., 1966; Fisher and Kaufman, 1972; Katz, 1980; Fitzpatrick, 1991). We have recently developed an alternative assay of aromatic amino acid hydroxylases, using oxymetric determination of reaction velocities (Fossbakk and Haavik, 2005). Using the oxymetric assay, we have been able to continuously measure the oxygen consumption catalyzed by TH and other hydroxylases. This procedure also allowed a correction for side reactions consuming oxygen and fast determination of initial reaction rates (<20 sec after addition of the enzyme) (Fossbakk and Haavik, 2005).

As shown in Fig. 2, a burst of oxygen consumption and TH activity was observed during the first few minutes of the enzyme reaction. Thus, 5 min after adding the enzyme, the hTH1 activity had declined to 31% of the initial rate. Adding supplementary oxygen (to 240 μM), tyrosine (to 50 μM) or BH_4 (to 200 μM) after 4 min incubation did not restore the enzyme activity, confirming that the rapid reduction in enzyme activity was not due to substrate depletion (data not shown). Similarly, the inclusion of 1 mg · ml^{-1} bovine serum albumin, 1 mg · ml^{-1} catalase, or 10% glycerol to the solution, or preincubation with dopamine (10 μM) did not protect the enzyme against inactivation. Furthermore, after increasing the TH concentration by adding a fresh aliquot of enzyme to the chamber, a high initial reaction rate and subsequent fall in activity was observed, suggesting that incubation conditions had not changed during the first minutes of the original enzyme reaction. Measurements of L-DOPA accumulation during this reaction interval showed a reaction stoichiometry of 1:1:1:1 between consumed tyrosine and dioxygen, and formation of ^3H_2O and L-DOPA (Fossbakk and Haavik, 2005).

Oxygen kinetics of isoenzymes of hTH, bovine TH and PAH

Both hTH3 and hTH4 displayed hyperbolic oxygen kinetics and rapid deactivation upon exposure to high

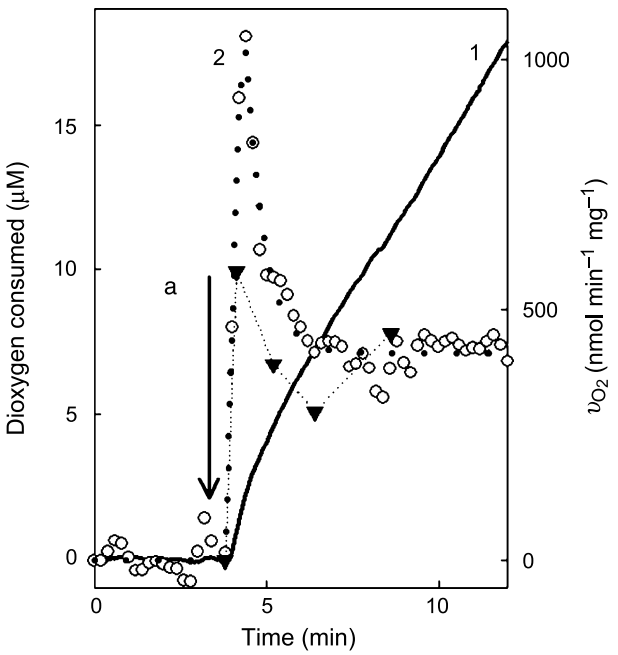

Fig. 2. Real time oxygraph recording of hTH activity. An oxygraph recording of the oxygen consumption during tyrosine hydroxylation is represented by the black trace (1), left axis, with the baseline change in oxygen concentration subtracted (see Materials and methods). The reaction is started by hTH addition, indicated by arrow a. The reaction velocity v_{O_2} (nmol · min^{-1} · mg^{-1}) is given by the open circles (2), right axis. For illustrative purposes, we included an interpreted curve to the data points (dotted line), where the decay in enzymatic activity going from a 'high-activity' state to a 'low-activity' state is shown by an exponential decay function (Eq. (2)). For comparison, the experimental data obtained using identical conditions in the ^3H_2O-release assay (Fig. 1A) have been plotted in the same axis (▼). The oxygen concentrations at the start of the experiments were 240 μM

Table 1. Kinetic values of the oxygen dependence of TH, Ser40-phosphorylated hTH1 (pS40-hTH1), and PAH obtained in this study and given in the literature

Enzyme source	$k_{cat}(O_2)$ (min^{-1})		$K_m(O_2)$ (μM)		Reference
	High-activity state	Low-activity state	High-activity state	Low-activity state	
Human TH1[a]	23.8 ± 2.0	17.3 ± 1.1	36.1 ± 2.3	16.2 ± 4.6	This work
Human TH1[b]	86.3 ± 2.9	16.7 ± 1.0	42.9 ± 3.8	15.9 ± 3.9	This work
Human TH3[b]	60.3 ± 2.3	29.4 ± 4.0	42.5 ± 8.3	26.7 ± 14.0	This work
Human TH4[b]	51.9 ± 3.9	11.1 ± 0.8	28.8 ± 7.7	12.6 ± 4.5	This work
pS40-hTH1[b]	76.5 ± 2.0	28.3 ± 1.0	40.0 ± 4.1	22.2 ± 3.9	This work
Rat TH (pure)[c]	–	75 ± 8	–	21 ± 19	Fitzpatrick (1991)
Rat TH (synaptosomes)	–	–	–	3–4[g]	Katz (1980)
Bovine TH (brain)	–	–	–	< ~11[e,h]	Fisher (1972)
Bovine TH (adrenal)	–	–	–	74[d]	Ikeda (1966)
Bovine TH (adrenal)	–	–	–	10–50[i]	Numata (1977)
Rat liver PAH	–	~61.6	–	<4[e,j]	Fisher (1972)
Human PAH[b,f]	–	99.2 ± 2.1	–	17.1 ± 2.9	This work

The time resolution of the radiochemical TH was lower than for the continuous oxymetric assay. In the radiochemical assay, the kinetic values for the high and low activity states were based on measurements obtained at 0.5–1 and 8 min, respectively. For convenience, literature values have been recalculated from partial pressures to molar concentrations of oxygen. Similarly, all enzymatic activities are presented as k_{cat}-values. Our experimental values are presented as means ± SEM, ($n = 10$–25)

[a] Radiochemical assay; [b] Oxygraphic assay; [c] 6-MPH$_4$ as cofactor; [d] 6,7-DMPH$_4$ as cofactor, oxygen concentration $7.4 \cdot 10^{-5}$ M; [e] Enzyme activity inhibited at oxygen concentrations above ~100 μM; [f] Without substrate activation; [g] Oxygen concentration 2–3 mmHg; [h] Oxygen concentration <1%; [i] Oxygen concentration 1–5%; [j] Oxygen concentration <0.35%

oxygen, as observed for hTH1. A similar reaction burst as for hTH was observed when hPAH was preincubated with phenylalanine, as described in Experimental procedures. Bovine TH and hPAH that had not been activated by phenylalanine before addition to the reaction mixture, had no apparent burst activities and lower K_m' for oxygen (Table 1). The more pronounced reaction burst observed in the oxymetric assay vs. the radiochemical assay may be due to the improved time resolution of the former assay.

Apparent K_m'- and k_{cat}-values were calculated from hyperbolic fitting to the rates measured at different oxygen concentrations following addition of the enzymes (Table 1). This gave results in concordance with the values obtained from the radioactive activity assays, but even more pronounced differences between early and late time points were observed. Thus, for hTH1 the K_m'-value decreased by more than 60% and the k_{cat}'-value by 80% going from the initial 0.5–1 to 8 min of enzyme assay (Table 1). The different isoforms of hTH did not show any dramatic differences in their kinetic character, as previously observed for the other substrates. However, hTH3 seemed to retain more of its activity, with less change in the K_m'-value throughout the length of the assay.

As in vivo activation, possibly by enzyme phosphorylation, of rat TH has been postulated to increase its oxygen affinity (Davis, 1976), it was of particular interest to determine the effects of TH phosphorylation. Figure 3 shows the oxygen dependence of initial enzyme activity in hTH1 in its nonphosphorylated and phosphorylated form. Phosphorylation at Ser-40 to a stoichiometry of 1 phosphate/monomer (Almas et al., 1992) did not alter the oxygen affinity of the enzyme (Table 1), but the phosphorylated enzyme retained more of its initial activity during incubation (Table 1).

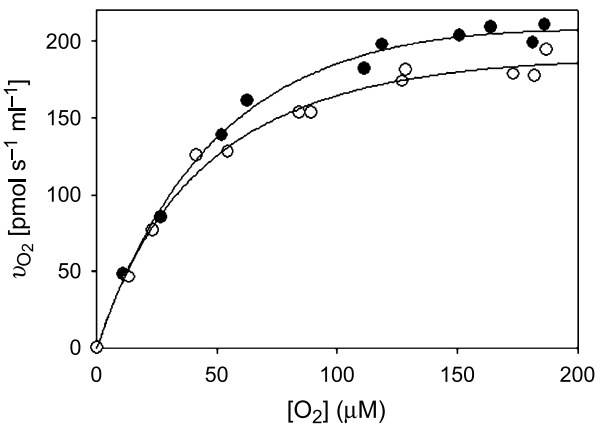

Fig. 3. Effect of Ser40 phosphorylation on the oxygen dependence of TH. The initial enzyme activity of hTH1 in its nonphosphorylated (●) and Ser40-phosphorylated (○) form (Experimental procedures) was measured at different dioxygen concentrations and the data were fitted to a hyperbolic equation

Oxygen dependent TH activity in intact PC12 cells

Having established consistently high K_m'-values of dioxygen for pure TH, it was also important to determine whether these values were relevant for intact catecholaminergic cells. Thus, we designed a cell culture based assay that could measure changes in TH activity less than 5 min after alterations in oxygen concentrations. For these studies, PC12 cells were selected as these cells have been considered a convenient model system for carotid body type 1 cells and also have been used extensively for studying molecular events occurring during hypoxic stress (Czyzyk-Krzeska et al., 1994).

Free 3,5-^{3}H-tyrosine added to the culture medium was rapidly taken up by the intact PC12 cells. Immediately after removal of extracellular tyrosine, the TH activity in the intact cells was determined by measuring the production of ^3H_2O from 3,5-^{3}H-tyrosine, before any further metabolic conversion of tyrosine had occurred. The TH activity was stimulated two-fold in the presence of an excess (100 μM) of extracellular BH_4, suggesting that the enzyme was not completely saturated by BH_4 in vivo. Furthermore, we found that the TH activity was dependent on the available concentration of O_2. The rates of ^3H_2O accumulation during a 5 min period were studied using different concentrations of dioxygen. When the oxygen concentration was raised from 33 to 139 μM, the rate of ^3H_2O accumulation increased by 115 ± 15% (mean ± SEM, $n = 5$–10, $p = 0.014$, ANOVA) (Table 2).

Redox state of the enzyme bound iron in the TH reaction

It is well established that the activity of the pteridine dependent aromatic amino acid hydroxylases is dependent on a non-heme iron(II) coordinated to His and Glu residues at the active site and that the oxidized versions of the enzymes are completely inactive (Wallick et al., 1984; Haavik et al., 1991; Ramsey et al., 1996; Goodwill et al., 1997; Kuhn et al., 1999). TH is typically isolated either as the inactive apoenzyme, or as Fe(III) forms with or without bound catecholamine inhibitors and is rapidly activated either by incorporation of Fe(II), by tetrahydropteridine mediated reduction of Fe(III), or by removal of the catecholamine inhibitors (Haavik et al., 1988). As it has been shown that a substoichiometrical oxidation of enzyme bound Fe(II) occurs during catalysis (Ramsey et al., 1996), we speculated whether this could explain the rapid decline in activity in the TH reaction. The redox state of the iron was examined using different iron chelators. The iron chelator desferrioxamine is a highly polar naturally occurring bacterial siderophore. Consistent with its selectivity for Fe(III), it shows no inhibitory action on TH1 during short-term incubations using the same experimental conditions as used in the oxygraphic measurements. However, in the presence of a large excess (65 μM) of desferrioxamine the reaction velocity gradually dropped to zero after a lag phase of 60–90 sec, consistent with a gradual trapping of Fe(III) by desferrioxamine and inhibition of its reincorporation/reduction into the enzyme. Desferrioxamine did not oxidize the free Fe(II) in the absence of TH. In contrast, the enzyme inhibition by Fe(II) chelators such as o-phenanthroline (Ramsey et al., 1996) or bathophenanthroline sulfonate (data not shown) occurred immediately after their addition to the enzyme. Assuming complete inhibition of the TH reaction upon oxidation to Fe(III), and a stoichiometry of 1.0 iron/enzyme subunit, it was calculated that approx. 16% of all TH turnovers resulted in conversion of the Fe(II) to Fe(III).

Table 2. Oxygen dependent TH activity in intact PC12 cells

Oxygen concentration (μM)	Activity (% of tyrosine converted · min^{-1})
32.9 ± 8.1	0.06 ± 0.02
138.9 ± 17.9	0.12 ± 0.02

The amount of ^3H_2O generated from ^{3}H-tyrosine was measured in suspensions of cells incubated in the oxygraph during exposure to different concentrations of dioxygen (see Materials and methods). The table shows the activity (% of tyrosine converted · min^{-1}) in samples where the oxygen concentration was kept at either 32.9 ± 8.1 μM or kept at 138.9 ± 17.9 μM O_2 for 20 min. The results are presented ± SEM ($n = 5$–10). The difference in the PC12 cell TH activity at low and high O_2 concentrations was significant ($p = 0.014$, ANOVA)

Discussion

Here we report for the first time the oxygen dependence of human TH. We have demonstrated high initial reaction rates in the hTH1 reaction, with initial K_m'-values of 42.9 ± 3.8 μM dioxygen (mean ± SEM, $n = 25$), indicating that the enzymatic activity may be severely limited by oxygen availability under such conditions. Similar values were obtained for hTH3 and 4. Moreover, we have described a turnover dependent inactivation of the enzyme using several independent assay procedures. Thus, for the different isoforms of TH we found a decrease in K_m' to 16–27 μM O_2 and a 2–5 fold reduction in the specific activity (Table 1). The large variation in apparent K_m values for oxygen in the previous studies may be due to methodological limitations. Thus, in former protocols the

concentrations of dioxygen in the experimental solutions have never been measured directly. Furthermore, TH has in most cases been extracted from mammalian tissues. The difficulty of obtaining adequate amounts of purified TH has probably contributed to the variable and conflicting results. In the present study we used a homogenous recombinant TH synthesized in *E. coli*. The most detailed previous kinetic characterization of TH has been performed by Fitzpatrick (1991, 2003). In the study on recombinant rat TH the apparent K_m for oxygen was reported to be $21 \pm 19\,\mu M$ (Fitzpatrick, 1991). Despite its uncertainty, this value is close to the average value of the ''low activity'' forms of the human enzymes. Thus, it is interesting that the properties of the rat enzyme synthesized in insect cells studied by Fitzpatrick (i.e. a eukaryote organism) are similar to the human enzymes synthesized in bacteria (Table 1).

The K_m'-values of the low activity forms of the hTH isoforms are closer to previous studies on TH from animals (Table 1). As the animal enzymes have been exposed to atmospheric levels of dioxygen, a partially oxidized and less active state of the enzyme may have been studied. Thus, the present measurements based on the initial reaction rate could give a better picture of the true TH oxygen kinetics in vivo. The finding of a rapid inactivation of the enzyme when it is exposed to supra physiological concentrations of oxygen, suggests that, ideally, enzyme kinetic studies should be carried out on enzymes not previously catalytically active, and in hypoxic chambers simulating tissue concentrations of dioxygen.

Inactivation of TH – enzyme kinetic considerations

We found a substantial decrease in TH activity during the first 1–2 min of the enzyme reaction. For both the oxymetric assay and the conventional TH assays that measure DOPA or ^3H_2O formation, the K_m-values for oxygen was also higher during the first minute of the reaction than later (Table 1). Our results strongly suggest that this is not caused by changes in substrate concentration or inhibitory products. Additionally, we found the same reaction pattern using L-phenylalanine as a substrate in the hTH reaction. Finally, the kinetic burst is not due to a slow dissociation of products from the enzyme, as the enzyme undergoes at least 70 turnovers per molecule during the burst phase and the reaction burst should not be regarded as a ''pre-steady state'' phenomenon in the conventional enzyme kinetic sense. Rather, our findings indicate that TH may exist in two different forms; a high k_{cat}/high K_m-state and a state with lower k_{cat}- and K_m-values. This is in accordance with recent findings of a negative cooperativity of BH_4-binding to hTH1 (Flatmark et al., 1999). Thus, the kinetic burst is most pronounced when the reaction is initiated by addition of fresh enzyme, as in this study.

The transition to a low-activity state could either be due to a slow conformational change or a covalent modification of the enzyme, e.g. oxidation of active site iron or redox sensitive amino acid residues. Evidence for both types of reactions has previously been presented for TH and other pterin dependent hydroxylases (Wallick et al., 1984; Ramsey et al., 1996; Kuhn et al., 1999). We also examined whether the inactivation could be due to a change in the oligomeric structure of TH following enzyme dilution. However, the rate of inactivation was not dependent on the concentration of protein added to the assay to the same final concentration of enzyme (0.048–4.8 mg/ml tested). However, the burst phase was more pronounced at 5 and 15 °C than at 25 °C, indicating a temperature dependent conversion between different enzyme forms (data not shown).

The enzyme oxidation hypothesis is supported by our finding that the inactivation rate was dependent on the initial oxygen concentration. Thus, at very low and probably physiological oxygen levels, there was little decline in activity. Incubating the enzyme in a solution with pure oxygen for several minutes without initiating the reaction, did not reduce the initial enzyme activity when, subsequently, the reaction was started by adding BH_4. Thus, the inactivation was turnover dependent. Furthermore, the putative oxidizing agent could be dioxygen itself, or a reactive oxygen species generated during catalytic turnover. The oxidizable target could include the enzyme active site Fe(II) or some critical sulfhydryl groups, which both have been shown to be important for catalytic activity and to be readily oxidized (Wallick et al., 1984; Kuhn et al., 1999). However, modification by N-ethylmaleimide of the sulfhydryl groups of hPAH did not alter the pattern of inactivation of that enzyme, indicating that sulfhydryl groups are not critical for this process (Fossbakk and Haavik, 2005). Preincubation of the enzyme with an excess of catalase, dopamine or protein stabilizing agents as glycerol and serum albumin did not prevent inactivation, confirming that it is not due to a non-specific protein modification, or due to hydrogen peroxide.

Interestingly, TH isolated from bovine adrenals had a lower initial activity and did not show the same pattern of turnover dependent inactivation. The latter enzyme, which already has been exposed to oxygen at atmospheric levels, and has been catalytically active, is probably in a reduced

activity form, $k_{cat}' = 23.6\,min^{-1}$ of bovine TH, compared to $k_{cat}' = 86.3\,min^{-1}$ for hTH1 (Haavik et al., 1988). Bovine adrenal TH is isolated in a partially phosphorylated state (0.62 ± 0.04 phosphate/subunit (Haavik et al., 1988)). Similarly, hTH1 that was phosphorylated on Ser40 retained more activity after oxygen exposure ($p < 0.05$, *t*-test) (Table 1). As phosphorylation of Ser40 is known to activate the enzyme and slightly change the ligand state of the active site iron (Andersson et al., 1992), we suggest that the iron site is also the main target of the turnover dependent inactivation of TH.

It is well established that TH containing Fe(II) is the catalytically active species (Haavik et al., 1991; Ramsey et al., 1996). Similarly, we have demonstrated that the enzyme bound Fe(II) in human TH can be oxidized to Fe(III) species by hydrogen peroxide or dioxygen, as studied by EPR, X-ray absorption, and Mössbauer spectroscopies (Meyer-Klaucke et al., 1996). The kinetics of Fe(II) oxidation in rat TH has been examined using a combination of visible and EPR spectroscopy and activity measurements (Ramsey et al., 1996; Frantom et al., 2006). In these studies, the loss of activity upon Fe(II) oxidation showed a close correlation with the appearance of a high-spin Fe(III) species by EPR spectroscopy and a visible absorbance band around 375 nm. A second order rate constant of $210\,M^{-1}\,sec^{-1}$ was determined for the oxidation of rat TH by oxygen (Frantom et al., 2006). During catalytic turnover, the regeneration of enzyme bound Fe(II) may become kinetically limiting. Thus, Frantom et al. have calculated the half-life of reduction of Fe(III)TH by BH_4 to be 3–5 sec under physiological conditions (Frantom et al., 2006). Ramsey et al. studied the effects of the Fe(III) chelator dihydroxynaphthalene on the rate of inactivation of rat TH, observing a similar time dependent inhibition of the enzyme as we observed with desferrioxamine (Ramsey et al., 1996). These findings indicate that a substantial amount of the active site Fe(II) is oxidized within the first 1–2 min of the enzyme reaction. As the kinetic burst cannot be overcome by increasing the exogenous concentrations of Fe(II) (Fig. 1B), we suggest that it is the reduction of active site Fe(III), rather than incorporation of ''new'' Fe(II) that becomes rate limiting during the steady state TH reaction. As the presence of iron chelators can perturb the Fe(II)–Fe(III) equilibrium, the use of a '' non invasive'' method such as EPR spectroscopy would be preferable (Andersson et al., 1992; Meyer-Klaucke et al., 1996). However, as this technique is relatively insensitive, it was not applied using the conditions of the enzyme kinetic measurements presented here.

Physiological implications

There have been many reports on tissue partial pressure of oxygen, generally showing that the oxygen concentration is low (Smith et al., 1977; Feinsilver et al., 1987; Siggaard-Andersen et al., 1995), and lower than the K_m-values for hTH reported here (Table 1). Thus, TH is probably not saturated with oxygen in vivo in humans. This is similar to human prolyl 4-hydroxylases which have $K_m(O_2)$-values of 230–250 μM, and where the enzyme activity has been shown to be directly proportional to tissue oxygen levels, making the enzymes effective oxygen sensors in vivo (Hirsila et al., 2003). Normobaric hypoxia decreases the accumulation of DOPA after the inhibition of aromatic L-amino acid decarboxylase, an index of in vivo tyrosine hydroxylation in rat brain (Davis and Carlsson, 1973; Brown et al., 1975; Katz, 1980). Similarly, we found an immediate decrease in ^3H_2O production from ^{3}H-tyrosine when PC12 cells were exposed to moderate hypoxia (Table 2). Studies in humans have also suggested a temporary reduction in catecholamine synthesis during hypoxia (Leuenberger et al., 1991; Sevre et al., 2001). Moreover, in humans the concentration of plasma catecholamines correlated well with the peripheral oxygen saturation (Rostrup, 1998). However, after longer exposure to hypoxia, the reduction in plasma catecholamine levels is no longer obvious (Rostrup, 1998). One explanation may be that other aspects of catecholamine metabolism are changed during hypoxia. Thus, peripheral clearance of circulating catecholamines is increased during short-term hypoxia (Leuenberger et al., 1991), while long-term effects on clearance have not been studied. Moreover, after a certain time compensatory mechanisms, such as enzyme induction, will take place (Gozal et al., 2005). The activity of other enzymes in the synthesis of catecholamines may also be sensitive to hypoxia. Thus, it has been suggested that the activity of dopamine-β-hydroxylase may be limited by hypoxia (Brown et al., 1975), which may further reduce the synthesis of noradrenaline and adrenaline.

Our results may be relevant for understanding the adaptation to hypoxic conditions in intact animals. In mammals, dopamine exerts a tonic inhibition of the carotid bodies. Thus, a reduced TH activity would lead to a diminished dopaminergic inhibition, causing a time-dependent increase in carotid body sensitivity to hypoxia (Bisgard, 1995). The rise in arterial chemoreceptor sensitivity at low oxygen pressures may therefore be an effect of reduced TH activity and reduced production of the inhibitor dopamine in the carotid bodies.

Conclusions

The K_m for dioxygen of hTH appears to be higher in the initial phase (1–2 min) of the enzyme activity in vitro than previously reported from animal studies. Tissue oxygen availability may be a limiting factor in the synthesis of catecholamines in humans. Furthermore, the enzyme is rapidly inactivated at the experimental conditions usually employed to measure in vitro activities. This could be due to the accumulation of an inactive Fe(III) form of the enzyme. The enzyme inactivation has important implications for the interpretation of kinetic data previously obtained on this enzyme.

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