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Biochemistry

"7-Tetrahydrobiopterin," a naturally occurring analogue of tetrahydrobiopterin, is a cofactor for and a potential inhibitor of the aromatic amino acid hydroxylases

(2-amino-4-hydroxy-7-[dihydroxylpropyl-(L-*erythro*)-5,6,7,8-tetrahydropterin])

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ABSTRACT **The ability of 2-amino-4-hydroxy-7-[dihydroxylpropyl-(L-*erythro*)-5,6,7,8-tetrahydropterin] ("7-tetrahydrobiopterin" or 7-BH_4) to substitute for the natural cofactor tetrahydrobiopterin (BH_4) has been studied *in vitro* in the reactions of the three mammalian aromatic amino acid hydroxylases. With rat liver phenylalanine hydroxylase, the apparent K_m for 7-BH_4 is 160 μM, a value that is ≈60-fold greater than that for the natural cofactor. In contrast, the hydroxylase reaction is severely inhibited by as little as 1 μM 7-BH_4 when assayed in the presence of physiological concentrations of BH_4. This inhibition can be overcome either by an increase in the concentration of BH_4 or a decrease in the concentration of phenylalanine. With both rat brain tryptophan hydroxylase and rat pheochromocytoma tyrosine hydroxylase, the K_m value for 7-BH_4 is about one order of magnitude greater than the K_m for BH_4. Accordingly, 7-BH_4 is a poor competitive inhibitor of both tryptophan and tyrosine hydroxylase. Thus, our results suggest that the observed hyperphenylalaninemia in patients who excrete 7-BH_4 in their urine may arise directly from the inhibition of phenylalanine hydroxylase by low levels of this pterin. On the other hand, it is less likely that low levels of 7-BH_4 would affect the activity of tyrosine or tryptophan hydroxylase *in vivo*.**

Hyperphenylalaninemia is an abnormality characteristic of a series of diseases that result from an inability to catalyze the first step in the catabolism of phenylalanine—namely, its conversion to tyrosine (1). The most common cause of this abnormality is the absence of active phenylalanine hydroxylase due to a defect in the gene coding for this enzyme (2, 3). Variant forms of this disease have been reported that are caused by genetic defects in either the biosynthesis of the essential pterin cofactor tetrahydrobiopterin (BH_4) or its enzymatic regeneration (4). Since BH_4 is required for catecholamine and serotonin biosynthesis by tyrosine hydroxylase and tryptophan hydroxylase, respectively (4), patients with these other forms of hyperphenylalaninemia also have a deficiency of biogenic amine neurotransmitters.

Recently, several patients have been described with a mild form of hyperphenylalaninemia, who have elevated urinary levels of an isomer of BH_4, 2-amino-4-hydroxy-7-[1,2-dihydroxypropyl)-(L-*erythro*)-5,6,7,8-tetrahydropteridine] ("7-tetrahydrobiopterin" or 7-BH_4),* and somewhat decreased levels of BH_4 (5). It has been suggested (6, 7) that the accumulation of abnormal amounts of 7-BH_4 might be the result of an alteration of the gene coding for 4a-hydroxytetrahydrobiopterin dehydratase (6, 7). This enzyme catalyzes the dehydration of 4a-hydroxytetrahydrobiopterin (4a-carbinolamine), the form of the pterin that is initially released from the aromatic amino acid hydroxylases after the BH_4-dependent hydroxylation of their respective substrates (7–9). *In vitro*, 4a-carbinolamine dehydrates at a rapid rate even in the absence of the dehydratase, but a small percentage of the 4a-carbinolamine rearranges to form the 7-isomer (6, 7). Thus the dehydratase plays the dual role of catalyzing the dehydration and preventing the isomerization of the pterin cofactor (7).

To date, the cause of the observed mild hyperphenylalaninemia in children who excrete 7-BH_4 remains unclear. Among the possible reasons for this abnormality are either that the reaction catalyzed by the dehydratase becomes rate-limiting due to the absence of the enzyme or that 7-BH_4 itself in some way impairs the phenylalanine hydroxylation reaction. One possible mechanism for such impairment became apparent when we showed that the oxidation of 7-BH_4 by phenylalanine hydroxylase in the presence of phenylalanine is 85–90% uncoupled from the hydroxylation of the substrate (10). In this report we demonstrate an additional mechanism by which 7-BH_4 might impair phenylalanine hydroxylase activity; i.e., 7-BH_4 is a potent inhibitor of this reaction. Furthermore, we have examined the effect of 7-BH_4 on the reactions catalyzed by tyrosine hydroxylase and tryptophan hydroxylase and have found that although these reactions are inhibited by this pterin *in vitro*, the inhibition is manifested only at very high concentrations of 7-BH_4.

MATERIALS AND METHODS

L-Phenylalanine, L-tyrosine, NADH, glucose 6-phosphate, glucose-6-phosphate dehydrogenase (from *Leuconostoc mesenteroides*), and *m*-hydroxylbenzylhydrazine were purchased from Sigma. L-Tryptophan and beef liver catalase were purchased from Calbiochem and Boehringer Mannheim, respectively. Frozen rabbit brains, used in the preparation of tryptophan hydroxylase, were purchased from Pel-Freez Biologicals. Sheep liver dihydropteridine reductase was purified through the calcium phosphate gel step (11). L-[3,5-^{3}H]Tyrosine (54 Ci/mmol; 1 Ci = 37 GBq) was from Amersham. 2-Amino-4-hydroxy-6-[dihydroxylpropyl-(L-*erythro*)-5,6,7,8-tetrahydropterin [(6*R*)-BH_4 or tetrahydrobiopterin], 6-methyltetrahydropterin (6MPH_4), and 7-BH_4 were purchased from B. Schirks Laboratories, Jona, Switzerland. All other reagents were of the highest quality available.

Phenylalanine Hydroxylase. Phenylalanine hydroxylase was purified from rat liver by a combination of two methods

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Abbreviations: BH_4, tetrahydrobiopterin; 7-BH_4, 2-amino-4-hydroxy-7-[dihydroxylpropyl-(L-*erythro*)-5,6,7,8-tetrahydropterin]; 6MPH_4, 6-methyltetrahydropterin.

*Due to the fluorescence properties of oxidized pterins, urine samples are oxidized prior to analysis by HPLC. For this reason, the actual compound that is detected in the urine of these patients by this method is 7-biopterin. However, it is probable that the 7-biopterin detected was derived from 7-BH_4.

as detailed (12). The hydroxylation of phenylalanine was monitored by either of two methods. The first measured the formation of tyrosine (13); the second used a spectrophotometric assay (14).

Tyrosine Hydroxylase. Pure tyrosine hydroxylase was obtained from cultured pheochromocytoma PC12 cells (15, 16). The hydroxylation of tyrosine was measured by the ^3H_2O release method (16, 17). The phosphorylation of tyrosine hydroxylase by cAMP-dependent protein kinase was performed as described (18), under conditions that resulted in the incorporation 0.6–0.7 mol of P_i per mol of enzyme subunit.

Tryptophan Hydroxylase. The hind and midbrain sections (including the superior and inferior colliculi and medulla oblongata) were dissected from whole rabbit brains and the tissue was homogenized in 3 vol of 50 mM Tris·HCl (pH 7.5) containing 0.1 M NaCl and 2 mM dithiothreitol. After centrifugation for 40 min at 110,000 × *g* (4°C), tryptophan hydroxylase was precipitated from the supernatant fraction by dropwise addition of a solution of 50% acetic acid to a final pH of 4.8. This mixture was stirred for 10 min at 4°C and the acidified mixture was centrifuged for 10 min at 12,000 × *g* (4°C). The pellet was resolubilized in homogenization buffer; this preparation served as the source of tryptophan hydroxylase used in these studies. The specific activity of this enzyme preparation was ≈0.35 nmol per min per mg. Tryptophan hydroxylase activity was measured by the fluorometric determination of 5-hydroxytryptophan (19).

RESULTS

The Kinetic Properties of Phenylalanine Hydroxylase with 7-BH_4. Phenylalanine hydroxylase catalyzes the hydroxylation of phenylalanine at a relatively slow rate in the presence of BH_4 (14). This rate can be substantially increased by preactivating the enzyme with its substrate, phenylalanine (20–22), or by exposure of the enzyme to certain phospholipids (23), such as lysolecithin (Table 1). In contrast, when 6MPH_4 is the cofactor, catalysis proceeds at a rapid rate with or without phenylalanine or lysolecithin preactivation. Similarly, these activation steps appear to have little effect on the rate of catalysis when 7-BH_4 is the cofactor; i.e., in this respect, the cofactor activity of 7-BH_4 is similar to that of 6MPH_4 rather than BH_4 (Table 1). Thus, the rates of the phenylalanine-dependent oxidation of BH_4, 6MPH_4, and 7-BH_4 by phenylalanine hydroxylase show only a small variance when the enzyme has been activated by either phenylalanine or lysolecithin, whereas the oxidation of BH_4 is 20- to 35-fold slower when the enzyme has not been activated (Table 1).

Table 1. Dependence of the initial rate of the phenylalanine-dependent tetrahydropterin oxidation on the activation of phenylalanine hydroxylase

Cofactor	Activator	Activity, μmol per min per mg	Fold activation
(6*R*)-BH_4	None	0.52	1
	Phenylalanine	12.4	24
	Lysolecithin	18.3	35
6MPH_4	None	21.2	1
	Phenylalanine	22.8	1.1
	Lysolecithin	26.0	1.2
7-BH_4	None	10.5	1
	Phenylalanine	12.8	1.2
	Lysolecithin	9.4	0.9

Phenylalanine-dependent tetrahydropterin oxidation by phenylalanine hydroxylase was measured spectrophotometrically. To test for the effect of phenylalanine activation, phenylalanine hydroxylase was preactivated at 26°C for 5 min with 1 mM phenylalanine prior to the assay. To minimize the effect of substrate inhibition on the reaction of phenylalanine hydroxylase with BH_4 in the presence of lysolecithin, the concentration of phenylalanine in the assay was 0.23 mM. All other assays with BH_4 contained 1 mM phenylalanine. For reactions containing 6MPH_4, 5 mM phenylalanine was included, whereas when 7-BH_4 was the cofactor 0.5 mM phenylalanine was included. The concentration of the cofactors BH_4, 6MPH_4, and 7-BH_4 were 100, 250, and 800 μM, respectively. With BH_4, the concentration of the hydroxylase was 6.6 or 3.3 μg/ml for the unactivated and activated enzyme reactions, respectively. With 7-BH_4 and 6MPH_4, enzyme at 1.7 μg/ml was added. (6*R*)-BH_4, 2-amino-4-hydroxy-6-[dihydroxylpropyl-(L-*erythro*)-5,6,7,8-tetrahydropterin].

Table 2. Comparison of the K_m values for the aromatic amino acid hydroxylases with BH_4 and 7-BH_4 as the cofactors

Hydroxylase	K_m (BH_4), mM		K_m (7-BH_4), mM	
	BH_4	A.A.	7-BH_4	A.A.
Phenylalanine	0.003	0.25	0.16	0.06*
Tyrosine	0.3	0.01*	2.2	0.15
Tryptophan	0.09	0.02*	1.7	ND

All apparent K_m values were determined by computer-fit to the Michaelis–Menten equation. K_m A.A. is the K_m value for phenylalanine, tyrosine, and tryptophan determined during their respective hydroxylations by their corresponding hydroxylases in the presence of either BH_4 or 7-BH_4. ND, not determined because of insufficient levels of activity and high substrate inhibition.

*K_m value listed is really the $S_{0.5}$ (the half-maximal substrate concentration). Substrate inhibition prevented the determination of a reliable K_m value.

The enzymatic oxidation of 7-BH_4 is ≈85% uncoupled (10), indicating that the rate of tyrosine formation in the presence of 7-BH_4 is ≈15% of the rate with BH_4 for the fully activated enzyme.† On the other hand, under conditions where phenylalanine hydroxylase has not been preactivated, the rate of tyrosine formation with 7-BH_4 as the cofactor is ≈3 times faster than with BH_4 as the cofactor.

The plot of the dependence of initial rate of tetrahydropterin oxidation on the concentration of 7-BH_4 with phenylalanine hydroxylase follows classical Michaelis–Menten kinetics with a K_m value 60-fold higher than the K_m determined for BH_4 under comparable conditions (Table 2). In contrast, the analogous plot obtained by varying the concentration of phenylalanine is complex (Fig. 1) and displays both cooperativity at low phenylalanine concentrations (*Inset*) and substrate inhibition at high phenylalanine concentrations. These kinetic characteristics were observed when the concentration of 7-BH_4 was maintained at 0.6, 1.0, and 6.9 times its K_m. Because of the complexity of the plot, the kinetic constants can only be estimated. A minimum value for the maximum velocity of the enzymatic oxidation of 7-BH_4 is ≈5 μmol per min per mg and the concentration of phenylalanine required for half-maximal activity is ≈60 μM. This apparent K_m for phenylalanine is ≈20% the value found when BH_4 is the

†Phenylalanine hydroxylase requires a tetrahydropterin cofactor to catalyze the hydroxylation of phenylalanine to tyrosine (24, 25). Since 1 mol of tetrahydropterin is oxidized per mol of phenylalanine hydroxylated when the cofactor is BH_4, the rate of the hydroxylation reaction has usually been monitored for the oxidation of NADH during the NADH-dependent regeneration of the tetrahydropterin by dihydropteridine reductase (14). A 1:1 stoichiometry is also observed when the cofactor analogue 6MPH_4 replaces BH_4 in the reaction (14). However, substitution of other cofactor analogues for BH_4 (e.g., 7-methyltetrahydropterin) can result in the uncoupling of the hydroxylation of the substrate from the oxidation of the cofactor (14). In these cases, phenylalanine hydroxylase partially behaves as a tetrahydropterin oxidase with the net oxidation of the tetrahydropterin exceeding the net hydroxylation of the substrate. The coupled and uncoupled oxidation of tetrahydropterins by phenylalanine hydroxylase appear to proceed by alternative pathways derived from a common enzyme-bound intermediate (26).

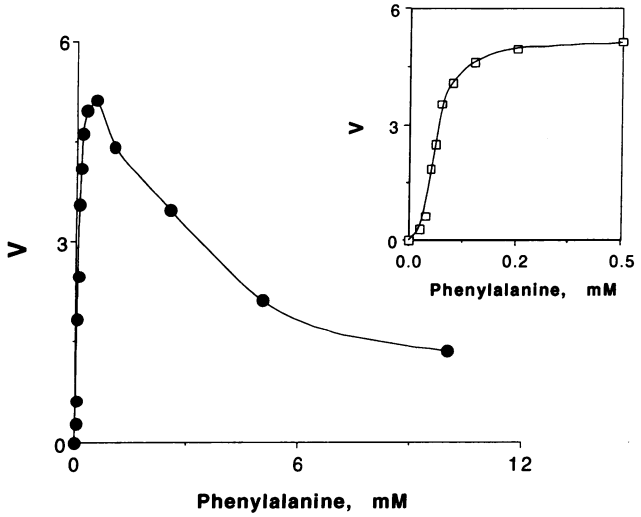

FIG. 1. Dependence of the initial rate of the phenylalanine hydroxylase-catalyzed reaction on the concentration of phenylalanine with 7-BH_4 as the cofactor. The assays were performed by monitoring the oxidation of NADH. *V* is the rate expressed in nmol of NADH oxidized per min per mg of enzyme. The concentration of 7-BH_4 was 760 μM and phenylalanine hydroxylase was present at 3.3 μg/ml. (*Inset*) Saturation curve for phenylalanine at low substrate concentrations.

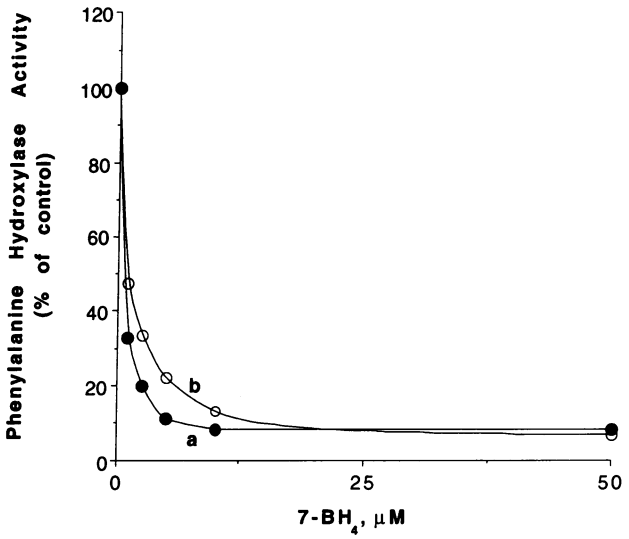

FIG. 2. Inhibition of nonactivated (curve a) and phenylalanine-activated (curve b) phenylalanine hydroxylase by various concentrations of 7-BH_4. The assays were monitored for tyrosine formation. Phenylalanine activation was performed as described in Table 1. The concentrations of phenylalanine and BH_4 were 0.9 mM and 5 μM, respectively. The 7-BH_4 concentration was varied as indicated and activated (4 μg/ml) or nonactivated (17 μg/ml) phenylalanine hydroxylase was added to the reactions. The data are expressed as a percentage of the full activity in the absence of 7-BH_4. The actual amount of tyrosine formed during the 30-min assay was 70 and 250 nmol for the reactions catalyzed by the nonactivated and the activated enzyme, respectively.

cofactor (Table 2). The plot of the dependence of initial rate on phenylalanine concentration obtained from direct measurements of tyrosine production had essentially identical characteristics to those in Fig. 1, except that the value of the rate of hydroxylation is ≈15% that of the cofactor oxidation as described above.

Inhibition of Phenylalanine Hydroxylase by 7-BH_4. Despite the relatively high apparent K_m for 7-BH_4, this pterin markedly inhibits the phenylalanine-catalyzed conversion of phenylalanine to tyrosine when BH_4 is the cofactor (Fig. 2). Even at a concentration as low as 1 μM 7-BH_4, an inhibition of >50% is observed in the presence of 5 μM BH_4 and saturating phenylalanine. This inhibition increased as a function of 7-BH_4 concentration. Preincubation of phenylalanine hydroxylase with phenylalanine to activate the enzyme only slightly decreased the ability of 7-BH_4 to inhibit the reaction (Fig. 2, curve b). On the other hand, increasing the concentration of BH_4 10-fold to 50 μM, (≈20 times the K_m) partially alleviated the inhibition (Fig. 3, compare curve c with curve d), as did decreasing the concentration of phenylalanine ≈5-fold to 200 μM (Fig. 3, compare curve c with curve b). Interestingly, a further decrease in the concentration of phenylalanine to physiological concentrations (≈60 μM) appeared to entirely prevent the inhibition by 7-BH_4 (Fig. 3, compare curve c with curve a).

The Kinetic Properties of Tyrosine Hydroxylase with 7-BH_4. Substitution of 7-BH_4 for BH_4 in the tyrosine hydroxylase-dependent hydroxylation reaction of tyrosine changes some of the kinetic properties of the enzyme (Table 2). Whereas the plot of the dependence of the initial rate on tyrosine hydroxylation, with BH_4 as the cofactor, is typically biphasic and displays severe substrate inhibition at tyrosine concentrations >50 μM (16, 27, 28), with 7-BH_4 as the cofactor, the plot is hyperbolic with no substrate inhibition at concentrations of tyrosine up to 300 μM (data not shown). Consequently, at high substrate levels, the rate of tyrosine hydroxylation with 7-BH_4 exceeds that measured with BH_4 (data not shown). The apparent K_m for tyrosine in the presence of 7-BH_4 is ≈13-fold higher than the half-maximal substrate concentration determined with the natural cofactor (Table 2). The apparent K_m for 7-BH_4, measured in the presence of 0.2 mM tyrosine (1.3 times the K_m), is 7-fold higher than the corresponding K_m for BH_4 with 20 μM tyrosine (2 times $S_{0.5}$, the half-maximal substrate concentration).

The most dramatic effect of phosphorylating tyrosine hydroxylase with cAMP-dependent protein kinase *in vitro* is a

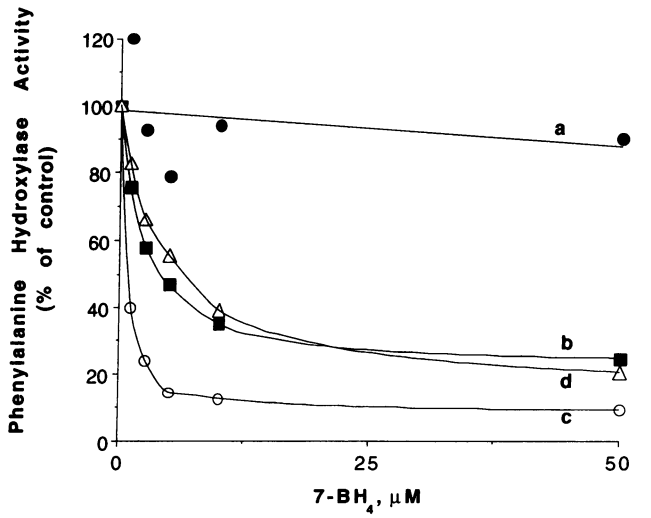

FIG. 3. Effect of increasing the concentration of BH_4 or decreasing the concentration of phenylalanine on the inhibition of phenylalanine hydroxylase by 7-BH_4. The assays were monitored for tyrosine formation. The concentration of 7-BH_4 was varied between 1 and 50 μM in samples containing 58 μM phenylalanine and 5 μM BH_4 (curve a), 230 μM phenylalanine and 5 μM BH_4 (curve b), 930 μM phenylalanine and 5 μM BH_4 (curve c), and 930 μM phenylalanine and 50 μM BH_4 (curve d). Nonactivated enzyme at 40 μg/ml (curve a) or 10 μg/ml (curves b–d) was added to each sample. The data are the percentages of the full activities measured in the absence of 7-BH_4, which were 3.8, 38, 83, and 68 nmol of tyrosine per 30 min for the conditions described in curves a–d, respectively.

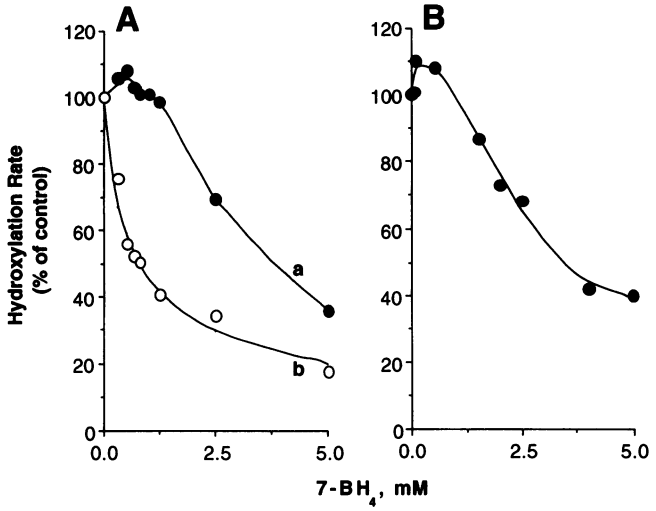

FIG. 4. Inhibition of tyrosine hydroxylase (*A*) and tryptophan hydroxylase (*B*) by increasing concentrations of 7-BH_4 in the presence of BH_4. The data are presented as the percentage of the full activity (control) measured in the absence of 7-BH_4. (*A*) Tyrosine hydroxylase was either unactivated (curve a) or activated (curve b) by previous phosphorylation with cAMP-dependent protein kinase. In each case, BH_4 was 100 μM and tyrosine was 25 μM. The full activities for the basal and phosphorylated enzymes were 110 and 240 nmol per min per mg, respectively. (*B*) Partially purified tryptophan hydroxylase was assayed with the indicated concentrations of 7-BH_4 in the presence of 100 μM BH_4 and 120 μM tryptophan. The full activity in the absence of 7-BH_4 was 0.33 nmol per min per mg.

decrease in the apparent K_m for BH_4 (29). This phenomenon is believed to play a major role in the regulation of the activity of the enzyme *in vivo* (29). When the activity was measured at pH 6, phosphorylation caused the expected decrease in the K_m for BH_4 from 300 μM to 100 μM, without a significant change in V_{max} (data not shown). A much more pronounced effect, however, was observed when 7-BH_4 was substituted for BH_4. The apparent K_m for 7-BH_4 decreased from 2.2 mM to $\approx$0.33 mM after phosphorylation, without an apparent change in V_{max} (data not shown). Accordingly, at physiological concentrations of BH_4 [$\approx$100 μM (30)], the phosphorylation of tyrosine hydroxylase decreased the IC_{50} of 7-BH_4 from 2.5 mM to $\approx$0.48 mM (Fig. 4A). Increasing the concentration of BH_4 to 250 μM caused a corresponding increase in the IC_{50} value to $\approx$700 μM, in agreement with the competitive nature of the inhibition by 7-BH_4 (data not shown).

The Kinetic Properties of Tryptophan Hydroxylase with 7-BH_4. In the presence of 0.2 mM tryptophan, the rate of the tryptophan hydroxylase-catalyzed conversion of tryptophan to 5-hydroxytryptophan with 7-BH_4 is only 5% that with the natural cofactor (data not shown). The apparent K_m for 7-BH_4 is 20-fold higher than for BH_4 (Table 2). The corresponding K_m for tryptophan with 7-BH_4 as the cofactor could not be determined due to the low rate of catalysis for this reaction and significant substrate inhibition at tryptophan levels >0.2 mM. 7-BH_4 is also a poor inhibitor of the hydroxylation of tryptophan by tryptophan hydroxylase with BH_4 as the cofactor. An IC_{50} for 7-BH_4 of 3.3 mM was determined when the enzyme was assayed in the presence of 0.12 mM tryptophan and 0.1 mM BH_4 (Fig. 4*B*).

DISCUSSION

The potent inhibition by 7-BH_4 of the phenylalanine hydroxylation reaction with BH_4 as the cofactor was somewhat surprising in view of the finding that the K_m for 7-BH_4 is 60-fold greater than that for the natural cofactor. However, at saturating levels of phenylalanine, concentrations of 7-BH_4 as low as 1 μM substantially inhibited the reaction with BH_4 even at concentrations of BH_4 equal to 2.5 times its K_m. Increasing the BH_4 concentration diminished the inhibition of 7-BH_4, implying that the inhibition is competitive. Decreasing the concentration of phenylalanine also alleviated the inhibition. In fact, at physiological concentrations of phenylalanine ($\approx$60 μM), even a 10-fold excess of 7-BH_4, relative to BH_4, failed to inhibit the hydroxylation reaction (Fig. 3, curve a). Further studies are required to determine whether the sensitivity of the 7-BH_4 inhibition at high phenylalanine concentrations is related to the substrate inhibition observed in the absence of BH_4.

The cause of the hyperphenylalaninemia observed in patients with an elevated excretion of 7-BH_4 is still uncertain. Our results suggest that the potent inhibition of phenylalanine hydroxylase by 7-BH_4 may play a role in the abnormal phenylalanine metabolism in these children. Although these patients have been classified as having mild hyperphenylalaninemia, it should be noted that they have blood phenylalanine levels between 5 and 20 times normal (31). Our *in vitro* results suggest that low concentrations of 7-BH_4 would have little effect on the rate of phenylalanine hydroxylation in the liver when phenylalanine is present at normal physiological levels, whereas significant inhibition of enzyme activity would occur at higher levels of phenylalanine. Therefore, restricting the intake of phenylalanine of hyperphenylalaninemic patients who excrete high concentrations of 7-BH_4 in their urine might be expected to prevent 7-BH_4 from inhibiting phenylalanine hydroxylation. Similarly, increasing BH_4 levels could also serve to counteract this inhibition, although to a lesser extent. This effect provides an explanation for the decrease in the blood phenylalanine levels of these patients when they are given BH_4 (5).

The effect of 7-BH_4 on the other two mammalian aromatic amino acid hydroxylases indicates that 7-BH_4 is a weak competitive inhibitor and a poor substitute for the natural cofactor in both hydroxylation reactions. With tyrosine hydroxylase, the apparent K_m for 7-BH_4 is decreased by phosphorylation and, therefore, increases competitiveness with BH_4. Nonetheless, even after phosphorylation, the high IC_{50} value for 7-BH_4 suggests that under physiological conditions, low levels of this pterin should have little effect on the rate of tyrosine hydroxylation. Similarly, our preliminary results obtained with tryptophan hydroxylase suggest that the activity of this enzyme also would not be affected by low levels of 7-BH_4 *in vivo*. Substantial inhibition of either of these enzymes could result in neurological defects arising from the impairment of the synthesis of those neurotransmitters derived from tyrosine or tryptophan (4). Such defects have not, however, been reported in the hyperphenylalaninemic patients with elevated levels of 7-BH_4 (5, 31).

It is currently believed that the increase in excretion of 7-BH_4 is due to a genetic defect in 4a-hydroxytetrahydrobiopterin dehydratase (6, 7). The dehydratase is predominantly expressed in three tissues: liver, kidney, and the pineal gland (32). Very little dehydratase activity, on the other hand, was found in the brain, where tryptophan hydroxylase and tyrosine hydroxylase are both expressed, or in the adrenals, where relatively high concentrations of tyrosine hydroxylase are found.‡ Since 4a-carbinolamine, the proposed precursor of 7-BH_4, has been shown to be the product of BH_4 oxidation

‡The pineal gland contains a relatively high concentration of tryptophan hydroxylase. Tryptophan hydroxylase from the pineal gland has several properties that differ from those of the brain enzyme. Thus, the finding of significant amounts of dehydratase activity in the pineal gland (32) suggests that further studies should be carried out to examine the kinetics of tryptophan hydroxylase from the pineal gland with 7-BH_4; 7-BH_4 may inhibit this enzyme more strongly than it inhibits the corresponding brain enzyme.

by tyrosine hydroxylase during the enzymatic conversion of tyrosine to dopa (33, 34), the low dehydratase activity in a tissue where tyrosine hydroxylase is found might result in the formation of some 7-BH_4. In normal individuals the amount of 7-BH_4 that presumably would be formed in the tissues with low dehydratase activity would likely be significantly less than the IC_{50} values determined for tyrosine and tryptophan hydroxylase and, thus, would not be expected to have a deleterious effect on either activity.

The plot of the dependence of initial rate on phenylalanine concentration with 7-BH_4 as the cofactor deviated substantially from a classical rectangular hyperbola, showing cooperativity at low phenylalanine concentrations and marked substrate inhibition at high phenylalanine concentrations (Fig. 1). The sigmoidal shape of the plot at low phenylalanine concentrations is similar to that reported for the analogous plot with BH_4 as the cofactor. For the natural cofactor, this apparent cooperativity has been postulated to be due to the need to activate phenylalanine hydroxylase with phenylalanine prior to catalysis and the ability of BH_4 to inhibit this activation (21, 22). The sigmoidicity of the plot, therefore, suggests that 7-BH_4 may also hinder the activation of the enzyme by phenylalanine.

Substrate inhibition with phenylalanine has been reported (23), previously, but to our knowledge, this is the first time it has been observed in the absence of some form of pharmacological preactivation of the enzyme. The substrate inhibition observed in Fig. 1 is also somewhat less marked than that observed previously in the presence of BH_4 (23). For example, the concentration of phenylalanine that allows the highest rate of catalysis (0.5 mM phenylalanine) is approximately twice that reported under the other conditions (23).

Although with the natural cofactor each of these deviations from classical Michaelis–Menten kinetics [i.e., sigmoidal kinetics and substrate inhibition (14)] have been reported individually, to our knowledge, these two characteristics have not been previously observed together. Thus, these kinetic characteristics of 7-BH_4 make it an ideal cofactor to probe certain aspects of the mechanism of phenylalanine hydroxylase.

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