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Role of C6 Chirality of Tetrahydropterin Cofactor in Catalysis and Regulation of Tyrosine and Phenylalanine Hydroxylases†

Steven W. Bailey, Shirley B. Dillard, and June E. Ayling*
Department of Pharmacology, College of Medicine, University of South Alabama, Mobile, Alabama 36688
Received April 2, 1991; Revised Manuscript Received July 22, 1991

ABSTRACT: The chiral specificities of bovine striatal tyrosine hydroxylase (TH) (unphosphorylated and phosphorylated by cAMP-dependent protein kinase) and rat liver phenylalanine hydroxylase (PH) were examined at physiological pH using the pure C6 stereoisomers of 6-methyl- and 6-propyl-5,6,7,8-tetrahydropterin (6-methyl-PH_4 and 6-propyl-PH_4) and (6*R*)- and (6*S*)-tetrahydrobiopterin (BH_4). Both PH and phosphorylated TH have substantially higher V_{max} values with the unnatural (6*R*)-propyl-PH_4 than the natural (6*S*)-propyl-PH_4 (approximately 6- and 11-fold, respectively). However, the K_m's are also higher such that V_{max}/K_m is almost unaffected by C6 chirality. Unphosphorylated TH has equal K_m values for both isomers of 6-propyl-PH_4, but has about a 6 times greater V_{max} with the unnatural isomer, making it the fastest cofactor yet for this form of the enzyme. With the shorter 6-methyl group, chiral differences are still recognized by phosphorylated TH but hardly at all by PH. Inhibition of both PH and TH by amino acid substrate which occurs with (6*R*)-BH_4 as cofactor is also observed with (6*S*)-propyl-PH_4 but not with (6*S*)-BH_4, (6*R*)-propyl-PH_4, or (6*R*)- or (6*R*,*S*)-methyl-PH_4. The K_m for (6*S*)-BH_4 with phosphorylated TH is nearly 3 times higher than with (6*R*)-BH_4, but V_{max} is unchanged. With unphosphorylated TH, (6*S*)-BH_4 produces very low decelerating rates, which was shown not to be due to irreversible inactivation of the enzyme. The K_m for (6*R*)-BH_4 with either hydroxylase is 10 times higher than for the equivalently configured (6*S*)-propyl-PH_4. Comparison of these two cofactors reveals that the 1′ and 2′ side-chain hydroxyl groups of the natural cofactor promote different regulatory functions in PH than in TH.

Although almost all of the function necessary to support the chemical steps of the aromatic amino acid hydroxylases reside in the tetrahydropteridine ring of their common cofactor (6*R*)-tetrahydrobiopterin [(6*R*)-BH_4][1] (Figure 1), it is becoming increasingly clear that the (6*R*)-L-*erythro*-dihydroxypropyl side chain is critical for many aspects of enzyme regulation (Kaufman & Fisher, 1974; Kaufman & Kaufman, 1985; Shiman, 1985; Bailey et al., 1990b). These phenomena,

† This investigation was supported by Grant GM-30368 awarded by the National Institute of General Medical Sciences and Grant NS-26662 awarded by the National Institute of Neurological Disorders and Stroke. Preliminary reports of part of this work have been presented (Bailey et al., 1990a,b).
* To whom correspondence should be addressed.

[1] Abbreviations: BH_4, tetrahydrobiopterin (6-[L-erythro-1′,2′-dihydroxypropyl]-5,6,7,8-tetrahydropterin); (6*R*)-BH_4, 6*R* diastereoisomer of BH_4 (the natural isomer); (6*S*)-BH_4, 6*S* diastereoisomer of BH_4 (the unnatural isomer); PH_4, tetrahydropterin; DOPA, dihydroxyphenylalanine; HPLC, high-pressure liquid chromatography; Na_2EDTA, ethylenediaminetetraacetic acid disodium salt; DHPR, dihydropteridine reductase.

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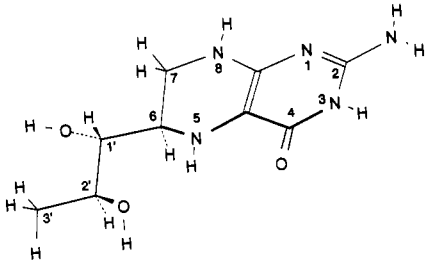

FIGURE 1: (6*R*)-Tetrahydrobiopterin, (6*R*)-[(1′*R*),(2′*S*)-dihydroxypropyl]-5,6,7,8-tetrahydropterin, (6*R*)-BH_4.

such as activation by substrate, inhibition by high substrate, and effects of protein phosphorylation, have generally been studied using mixtures of (6*R*) and (6*S*) tetrahydropterin enantiomers. The exception has been the natural cofactor itself, (6*R*)-BH_4, which has been compared with the (6*S*) isomer with phenylalanine hydroxylase (Bailey & Ayling, 1978a; Haavik et al., 1986), tyrosine hydroxylase (Oka et al., 1981), and tryptophan hydroxylase (Hasegawa et al., 1979; Kato et al., 1980a). All three hydroxylases respond quite differently to (6*S*)-BH_4 and (6*R*)-BH_4. With phenylalanine hydroxylase, inhibition by high phenylalanine is dependent on the presence of the *R* configuration (Bailey & Ayling, 1978a). Further, with this hydroxylase the natural cofactor more potently inhibits cAMP-dependent phosphorylation (Døskeland et al., 1987). The unnatural isomer of tetrahydrobiopterin supports slower maximum velocities with rat phenylalanine, and tryptophan hydroxylases, and causes an irreversible inactivation of the fomer (Parniak & Kaufman, 1983). The cofactor properties of (6*S*)-BH_4 with tyrosine hyroxylase have been studied only with unphosphorylated enzyme and at pH 6 (Oka et al., 1981), both conditions having considerable impact on kinetic parameters (Miller & Lovenberg, 1985; Bailey et al., 1989). The influence of tetrahydrobiopterin 6-position stereochemistry on this hydroxylase at physiological pH has not been previously investigated.

The purpose of this investigation was to ascertain the functional significance of 6-position stereochemistry and the two hydroxyl groups in the side chain of tetrahydrobiopterin. This required pure (6*R*) and (6*S*) isomers of BH_4 analogues. Resolution of the two BH_4 isomers, which can be achieved by either chromatographic separation (Bailey & Ayling, 1978a; Matsuura et al., 1985a), fractional crystallization (Ganguly & Viscontini, 1982), or stereoselective reducton (Matsuura et al., 1985b) is aided by the diastereomeric nature of the dihydroxypropyl side chain (Figure 1). So far, of the cofactor analogues lacking chiral centers within the side chain, only (6*R*,*S*)-methyltetrahydropterin has been resolved through the fractional crystallization of a diastereomeric salt (Armarego, 1979). Dihydrofolate reductase can be used to selectively reduce 7,8-dihydropterins, but this does not provide the unnatural isomer. This deficiency of 6-position isomers has been overcome by the development of a direct chiral synthesis of tetrahydropterins capable of producing a wide variety of analogues of either chirality having greater than 99% enantiomeric purities. This method was employed to produce (6*R*)-methyl-, (6*S*)-propyl-, and (6*R*)-propyltetrahydropterins. These, as well as (6*S*)-BH_4 and (6*R*)-BH_4, have been used to probe the enantiomeric specificity of phenylalanine and tyrosine hydroxylases at physiological pH. The kinetics of mixtures of these cofactor enantiomers were also examined to allow previous results obtained with racemates to be compared with the enzymatic behavior of the pure natural isomers. The results show that C6 stereochemistry can have a major influence even in the absence of other chiral centers.

Materials and Methods

Enzymes. Phenylalanine hydroxylase from rat liver was purified by the method of Shiman et al. (1979). Tyrosine hydroxylase was a partially purified extract of bovine brain striata, treated with sodium borohydride to inactivate contaminating DOPA decarboxylase, as described previously (Bailey et al., 1989). Dihydropteridine reductase (DHPR) from bovine liver was purified and assayed as previously described (Bailey & Ayling, 1983). C4a-Carbinolamine dehydratase (Lazurus et al., 1983) was purified 200-fold from rat liver by the method of Huang et al. (1973), except the phosphocellulose column chromatography step was omitted. Bovine erythrocyte superoxide dismutase and cAMP-dependent protein kinase from bovine heart were purchased from Sigma. Bovine liver catalase was from Worthington (LS00-01898).

Reagents. (6*R*,*S*)-Methyltetrahydropterin and (6*R*)- and (6*S*)-tetrahydrobiopterin were obtained from. Dr. B. Schircks, Jona, Switzerland. The (6*R*)-BH_4 was found to contain less than 0.5% of the (6*S*) isomer and was used without further purification. The (6*S*)-BH_4 was purified, when necessary, of contaminating (6*R*)-BH_4 by a modification of a published procedure (Bailey & Ayling, 1978a). This employed the more retentive RSil SCX (10 μm, 1 × 25 cm) cation exchanger (Alltech Assoc.), eluted with ammonium formate, pH 3.3 (0.2 M in ammonia), at 3.0 mL/min at a column temperature of 4 °C. The column was first treated by an injection of $Na_2S_2O_4$ as described in the above reference. The lower temperature has a marked influence on increasing the resolution of the two 6-position diastereomers, a phenomenon possibly linked to differences in intramolecular hydrogen bonding.[2] The improved separation, primarily due to increased k' for (6*S*)-BH_4, far outweighs the slight loss of plate number and allows recovery of this isomer completely free of the earlier eluting (6*R*)-BH_4. (6*R*)-Methyl-, (6*R*)-propyl-, and (6*S*)-propyltetrahydropterins[3] were synthesized by a new procedure to be published elsewhere.[4] Analysis of each compound by HPLC showed that greater than 99% of the total absorbance at 265 nm and the electrochemical response at +0.30 V versus Ag/AgCl resided in a single peak. All were also determined to be greater than 99.5% enantiomerically pure by chiral HPLC. (*m*-Hydroxybenzyl)hydrazine dihydrochloride, L-phenylalanine, L-tyrosine, and L-DOPA were from Sigma.

6,7-Dihydroxy-3-carboxy-1,2,3,4-tetrahydroisoquinoline was prepared by a modification of the method of Bell et al. (1971) in which 500 mg of L-DOPA (2.54 mmol) was stirred as a suspension with 0.5 mL of 37% formaldehyde (6.15 mmol) in 25 mL of H_2O at room temperature for 23 h under argon. The mixture was then placed on ice for several hours and then centrifuged, and the precipitate was washed with 2 mL of ice-cold water and dried under vacuum over P_2O_5 to give 233 mg of white powder. The remaining supernate contained a substantial amount of product contaminated with traces of starting material and several other compounds. HPLC analysis of the collected product using Adsorbosphere ODS, 3 μm, 15 × 0.41 cm (Alltech Assoc.) eluted with 600 mg/mL octanesulfonic acid and 1 mM Na_2EDTA in ammonium formate, pH 3.3 (30 mM in ammonia), at 1 mL/min showed the main peak to represent 98% of the total UV_{280} absorbance and 99.7% of the fluorescence (282-nm excitation, 325-nm emission). The

[2] M. Mezei, S. W. Bailey, and J. E. Ayling, manuscript in preparation.

[3] According to the Cahn, Prelog, Ingold designation, the natural configuration of 6-alkyltetrahydropterins (lacking the 1′-hydroxyl) is 6*S*.

[4] S. W. Bailey, and J. E. Ayling, manuscript in preparation.

only fluorescent impurity appeared to be due to a 1% contamination with DOPA. [UV (0.1 N HCl) λ_{max} = 285 nm, ϵ = 3400 M^{-1} cm^{-1}.]

Hydroxylase Assays. Tyrosine hydroxylase is phosphorylated by several different protein kinases, but by far the largest effect is seen with cAMP-dependent protein kinase which phosphorylates serine-40 of the bovine enzyme (Haavik et al., 1989), as with the rat pheochromocytoma enzyme (Campbell et al., 1986). This phosphorylation decreases K_m for cofactor analogues by 3 orders of magnitude (Bailey et al., 1989). Therefore, the effect of C6 chirality of cofactors was determined with both "unphosphorylated" (i.e., not specifically treated with a protein kinase) and "phosphorylated" (i.e., exposed to continuous high activity of cAMP-dependent protein kinase) tyrosine hydroxylase. All reactions were performed at pH 7.15 and 37 °C in a total volume of 0.1 mL as previously described (Bailey et al., 1989) except that (*m*-hydroxybenzyl)hydrazine was included to further suppress dopamine formation beyond that provided by sodium borohydride inactivation of DOPA decarboxylase (see above). This was found to be necessary in reactions in which large quantities of DOPA were formed.

Unphosphorylated tyrosine hydroxylase was assayed as previously described except for some reactions of 6-propyl-PH_4 in which 0.1 μmol of NADH and 0.1 unit of dihydropteridine reductase were added, instead of sodium ascorbate, just before tyrosine hydroxylase to begin the 10-min preincubation period. After initiation of the reaction with tyrosine (unless otherwise specified), aliquots were removed at timed intervals for 30 min and quenched in 2 volumes of 0.75 M trichloroacetic acid. The amount of tyrosine hydroxylated in each sample is reported as the sum of the DOPA and 6,7-dihydroxy-3-carboxy-1,2,3,4-tetrahydroisoquinoline, both of which were separated by HPLC, (Spherisorb ODS II, 3 μm, 15 × 0.46 cm, with ammonium formate, pH 3.3 (30 mM in ammonia), containing 1 mM Na_2EDTA as the mobile phase), detected fluorometrically (282-nm excitation, 325-nm emission), and quantitated by comparison to standards (Bailey et al., 1989). In this system the latter compound elutes earlier than DOPA and produces about 3–4 times more fluorescence response (peak height) per nanomole than DOPA.

Phosphorylated tyrosine hydroxylase assays were assembled in a manner similar to that previously reported (Bailey et al., 1989) such that the hydroxylase is maintained at 90% or greater phosphorylation throughout both the preincubation and reaction periods. Standard reactions were at a final pH of 7.15 at 37 °C and contained (per 0.1-mL final volume) 20 nmol of tyrosine [except 10 nmol with (6*R*)-BH_4, or when the varied substrate], 2 μmol of sodium ascorbate, 25 units of superoxide dismutase, 250 units of catalase, 10 μmol of potassium phosphate, pH 7.18 (initial pH before addition of any other components), 0.5 nmol of (*m*-hydroxybenzyl)hydrazine, 0.2–1.0 nmol unit of cAMP-dependent protein kinase (depending on the amount of tyrosine hydroxylase), 0.5 μmol of $MgCl_2$, 0.5 nmol of cAMP, tyrosine hydroxylase, and 0.12 μmol of ATP. After the addition of ATP as the next to last component, the mixture was preincubated at 37 °C for 10 min, and the reaction was initiated by addition of tetrahydropterin cofactor. Aliquots were removed at timed intervals for 30 min, added to 2 volumes of 0.75 M trichloroacetic acid, and analyzed for DOPA as discussed above for unphosphorylated reactions.

Phenylalanine hydroxylase reactions were monitored spectrophotometrically at 340 nm for the consumption of NADH in the presence of excess DHPR. Both isomers of quinoid-6-propyldihydropterin were determined to be good substrates for the reductase as will be reported elsewhere.[4] Standard reactions contained, unless otherwise specified, 0.1 mmol of Tris-HCl, pH 7.4, at 27 °C, 80 μg (240 units) of superoxide dismutase, 2500 units of catalase, 1 μmol of L-phenylalanine (when not the varied substrate), 10 μg of C4a-carbinolamine dehydratase, 0.4 units of dihydropteridine reductase, and betweeen 6 and 12 milliunits of phenylalanine hydroxylase. These reactants were preincubated together in a cuvette for 5 min at 27 °C before addition of 0.1 μmol of NADH. The reaction was then initiated with tetrahydropterin cofactor to make a total volume of 1.0 mL. Tetrahydropterin cofactors (as dihydrochloride salts) were dissolved in argon-saturated water in concentrations such that less than 75 μL was added to the reaction, so that near equilibration with atmospheric oxygen was maintained. The stoichiometry of tyrosine production to cofactor consumption was determined by removing 20-μL aliquots from the cuvette every 30 s and quenching with 20 μL of 1.0 M trichloroacetic acid. These samples were centrifuged at 14000*g* for 5 min, and the supernates were analyzed for tyrosine by HPLC with fluorescence detection as previously described (Bailey & Ayling, 1980), except for the substitution of Spherisorb ODS II, 5 μm, 15 × 0.46 cm (Alltech Assoc.) for the former nonendcapped Spherisorb ODS I. The stoichiometry was determined not only from comparison of initial rates of 340-nm absorbance changes with initial rates of tyrosine formation but also from the slopes of parametric plots of tyrosine formed vs NADH consumed along the progress of reactions for approximately 5 min. Phenylalanine hydroxylase, as purified, contains 0.2–0.3 phosphates per 50000 kDa subunit, which can be increased to 1 phosphate per subunit by treatment with cAMP-dependent protein kinase. This treatment was found to have only a small effect on the activity of phenylalanine hydroxylase, in agreement with earlier investigations (Abita et al., 1976). Exposure of the purified enzyme to phosphatases has no effect on its activity. Therefore, all studies with phenylalanine hydroxylase were done with the enzyme as purified.

Data Analysis. Kinetic constants were calculated with a PC-compatible nonlinear regression program (MINSQ; Micromath, Salt Lake City, Utah) as previously described (Bailey et al., 1989).

Results

Tyrosine Hydroxylase

Progress Curves. Reactions of unphosphorylated tyrosine hydroxylase that are initiated with cofactor show a lag which lasts 5–10 min depending on cofactor structure and concentration. Preincubation with cofactor for 10 min at 37 °C prior to initiation of the reaction with tyrosine decreased the lag as illustrated with tetrahydrobiopterin in Figure 2 (upper two curves). Rates were obtained from the fastest portion of the progress curves. The rate of DOPA formation in reactions with above 1.5 mM (6*R*)- or (6*S*)-propyl-PH_4 decelerated considerably after 10 min. A contribution to this effect was found to be the non-enzymatic conversion of part of the DOPA into 3-carboxy-6,7-dihydroxytetrahydroisoquinoline in the later part of the reaction. The extent to which this compound is formed depends on the structure of the tetrahydropterin cofactor and is especially noted at high cofactor concentrations and after long reaction times. Tetrahydroisoquinoline formation has been shown to be due to the liberation of formaldehyde as a decomposition product of quinoid and 7,8-dihydropterins and its subsequent reaction with DOPA (Ayling et al., 1990b). Progress curves were, therefore, corrected to

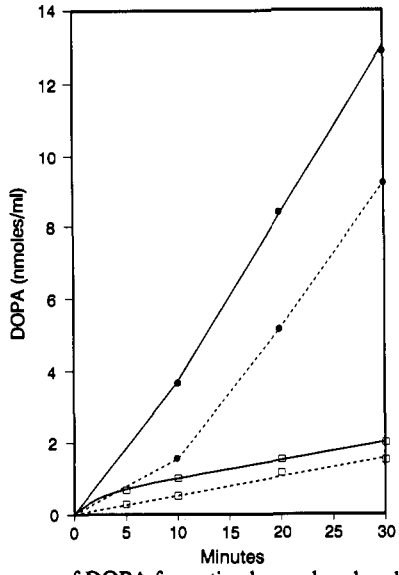

FIGURE 2: Progress of DOPA formation by unphosphorylated tyrosine hydroxylase with (*R*)-BH_4 (●, upper 2 curves) and (6*S*)-BH_4 (□, lower two curves), both at 3 mM and in the presence of 0.1 mM tyrosine at 37 °C in 0.1 M KPO_4, pH 7.15. Reactions were either preincubated with cofactor for 10 min at 37 °C and initiated with tyrosine (solid lines) or preincubated with tyrosine for 10 min and initiated with cofactor (dashed lines). The reactions with (6*S*)-BH_4 contained 4 times more hydroxylase than those with (6*R*)-BH_4.

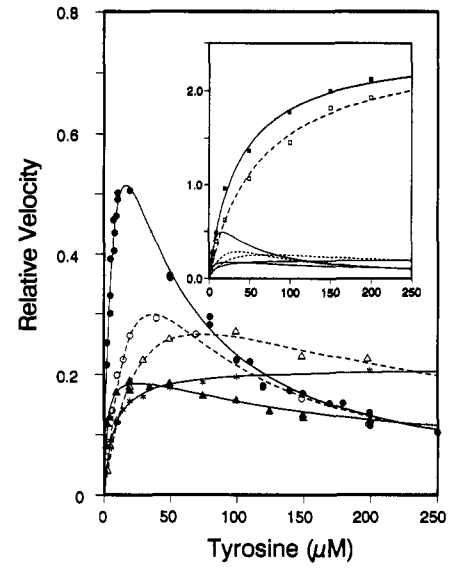

FIGURE 3: The rate of L-DOPA formation by tyrosine hydroxylase both unphosphorylated (dotted lines with open symbols) and phosphorylated by cAMP-dependent protein kinase (solid lines) at 37 °C in 0.1 M KPO_4, pH 7.15, as a function of tyrosine concentration. The cofactors were (6*R*)-BH_4 (●, ○), (6*S*)-BH_4 (*), (6*S*)-propyl-PH_4 (▲, △), and (6*R*)-propyl-PH_4 (■, □), all used at the concentrations specified in Table I. Rates are relative to the V_{max} of phosphorylated tyrosine hydroxylase with 0.5 mM (6*R*,*S*)-methyl-PH_4, which is defined as 1. The inset contains the same data as in the main figure, but with a 3-fold greater range on the *y* axis so that rates with (6*R*)-propyl-PH_4 can be shown.

reflect the total of both products. Although related tetrahydroisoquinolines have been reported to be inhibitors of tyrosine hydroxylase (Weiner & Collins, 1978; Bennett et al., 1982), this does not appear to be a factor in the current study. The level of 3-carboxy-6,7-dihydroxytetrahydroisoquinoline produced in any reaction did not exceed 1 μM, and yet even 25 μM added to the reaction did not inhibit either phosphorylated or unphosphorylated tyrosine hydroxylase [100 μM tyrosine; 3.0 μM and 3.0 mM (6*R*)-BH_4, respectively].

The reactions of both isomers of 6-propyl-PH_4 with unphosphorylated tyrosine hydroxylase were found to be unusual in comparison to those reported previously for other cofactors (Bailey et al., 1989) in that 20 mM ascorbate proved inadequate to maintain high concentrations of this cofactor in the reduced form, resulting in an apparent inhibition above 2 mM cofactor. This problem was overcome by the use of NADH and a high activity of dihydropteridine reductase which efficiently reduces both isomers of quinoid-6-propyl-PH_2.[4] The rates of DOPA formation using the ascorbate and NADH/DHPR regenerating systems agree at low 6-propyl-PH_4 concentration, but only the latter was capable of maintaining hyperbolic kinetics above 2 mM.

The shape of progress curves of phosphorylated tyrosine hydroxylase with (6*R*)-methyl or either isomer of 6-propyl-PH_4 are qualitatively similar to reactions with other (6*R*,*S*)-alkyltetrahydropterins at pH 7.15 (Bailey et al., 1989). With each of these cofactors the rate decreases continuously with time. This deceleration is in contrast to reactions with the natural cofactor (6*R*)-BH_4 which are linear for at least 30 min. Progress curves with (6*S*)-BH_4 as cofactor were qualitatively similar to those with the 6-alkyltetrahydropterins, although the deceleration with time was significantly faster. Insignificant quantities of 3-carboxy-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline are formed in any phosphorylated tyrosine hydroxylase reaction due to the low concentratons of cofactor required for saturation. Kinetic parameters were determined from the initial rate of DOPA formation.

K_m for Tyrosine. Michaelis–Menten curves for tyrosine showed an unexpected inhibition at high substrate with the (*S*) isomer of 6-propyl-PH_4 as cofactor. Whereas (6*R*)-propyl-PH_4 produced hyperbolic plots, the natural (*S*) configuration[3] gave rates which declined at higher tyrosine with unphosphorylated and to an even greater extent with phosphorylated tyrosine hydroxylas (Figure 3). The steepness of the rise at low concentrations in the curve with phosphorylated enzyme is similar to that of (6*R*)-BH_4, but inhibition at high substrate is not as pronounced. Also, with phosphorylated hydroxylase, a 1:1 mixture of (6*R*)- and (6*S*)-propyl-PH_4 resulted in a tyrosine saturation curve similar in shape to that for the pure (6*S*)-propyl-PH_4, except for a slightly increased maximum observed rate. No substrate inhibition was observed at neutral pH with phosphorylated or unphosphorylated tyrosine hydroxylase with (6*S*)-BH_4 (Figure 3), (6*R*)-methyl-PH_4, or (6*R*,*S*)-methyl-PH_4 as cofactor. A lack of substrate inhibition at pH 6 with the unnatural isomer of tetrahydrobiopterin has previously been reported with unphosphorylated tyrosine hydroxylase (Oka et al., 1981). Considering the lower K_m for the natural (6*R*) isomer of tetrahydrobiopterin, it is not surprising (see discussion of eq 1) that a 2:1 (6*R*)/(6*S*) mixture typical of catalytically reduced material (Bailey & Ayling, 1978) gave a pattern of tyrosine substrate inhibition (data not shown) very similar to that with pure (6*R*)-BH_4 (Figure 3). The inhibition at high tyrosine previously reported with (6*R*,*S*)-BH_4 as cofactor for unphosphorylated hydroxylase at pH 6 (Shiman et al., 1971; Numata et al., 1977) appears more pronounced than the current result, perhaps indicating a pH dependence of this phenomenon.

Although (6*S*)-BH_4 does not induce substrate inhibition, comparison with (6*R*)-propyl-PH_4 (i.e., matching C6 chirality) shows a K_m for tyrosine with phosphorylated tyrosine hydroxylase that is decreased by a factor of 6 due only to the presence of the 1′- and 2′-hydroxyl groups (Table I). This is consistent with the hypothesis of Kato et al. (1980b), who speculated from studies with cofactor analogues racemic at C6 that the presence and chirality of the 1′-hydroxyl group

Table I: Effect of Cofactor C6 Chirality on the K_m for Tyrosine with Tyrosine Hydroxylase

	unphosphorylated			phosphorylated[a]		
cofactor/isomer	cofactor concn (mM)	tyrosine K_m (μM)	V_{max}[d]	cofactor concn (mM)	tyrosine K_m (μM)	V_{max}[d]
6-propyl-PH_4[b]						
(*S*)	1.4	19–60[e]	>0.26[c]	0.02	2–8[e]	>0.2[c]
(*R*)	1.4	60	2.5	0.1	40	2.5
1:1 (*R*)/(*S*)				0.02	3–10[e]	>0.3[c]
6-methyl-PH_4[b]						
(*R*)				0.5	60	3
(*R,S*)	5	60	0.18	0.5	45	1
BH_4						
(*R*)	5	12–80[e]	>0.3[c]	0.05	5–30[e]	>0.5[c]
(*S*)		*f*	*f*	0.075	7	0.22
2:1(*R*)/(*S*)				0.05	5–30[e]	>0.3[c]

[a] Phosphorylated by cAMP-dependent kinase. [b] Both (6*S*)-propyl-PH_4 and (6*S*)-methyl-PH_4 have the same C6 chirality as the natural isomer of tetrahydrobiopterin, (6*R*)-BH_4. [c] Substrate inhibited by tyrosine; values represent maximum observed rate. [d] Maximum velocities are relative to the V_{max} with 0.5 mM (6*R,S*)-methyl-PH_4 with phosphorylated tyrosine hydroxylase, which is defined as 1. [e] The range of K_m calculated from best fits of the entire saturation curve to five different mechanisms of substrate inhibition. [f] The kinetic parameters of unphosphorylated tyrosine hydroxylase with (6*S*)-BH_4 cannot be quantitated (see text).

Table II: Kinetic Parameters for Cofactor C6 Stereoisomers with Tyrosine Hydroxylase

	unphosphorylated			phosphorylated[a]		
cofactor/isomer	cofactor K_m (mM)[b]	V_{max}[e]	V_{max}/K_m	cofactor K_m (mM)[b]	V_{max}[e]	V_{max}/K_m
6-propyl-PH_4[c]						
(*S*)	0.6	0.34[d]	0.6[d]	0.0003	0.17[d]	570[d]
(*R*)	0.6	2.1	3.5	0.005	1.8	360
1:1 (*R*)/(*S*)	0.6	0.5	0.8	0.0004	0.21[d]	525[d]
1:1 (*R*)/(*S*) calcd[f]	0.6	1.2	2.0	0.0005	0.26	520
5:1 (*R*)/(*S*)				0.0016	0.46	290
5:1 (*R*)/(*S*) calcd[f]				0.0014	0.54	385
6-methyl-PH_4[c]						
(*R*)				0.13	3.3	25
1:1 (*R*)/(*S*)	3	0.3	0.1	0.06	1	17
(*S*) calcd[f]				0.04	0.3	8
BH_4						
(*R*)	~5	~0.5[d]	0.1[d]	0.003	0.24[d]	80[d]
(*S*)	*g*	*g*		0.008	0.24	30
2:1 (*R*)/(*S*)				0.004	0.22[d]	55[d]
2:1 (*R*)/(*S*) calcd[f]				0.004	0.24	60

[a] Phosphorylated by cAMP-dependent protein kinase. [b] Apparent K_m's for cofactor were measured at 0.2 mM tyrosine, except with (6*R*)- and (6*R,S*)-BH_4, where 0.1 mM was used. [c] Both (6*S*)-propyl-PH_4 and (6*S*)-methyl-PH_4 have the same C6 chirality as the natural isomer of tetrahydrobiopterin, (6*R*)-BH_4. [d] Substrate inhibited by tyrosine. [e] Maximum velocities are relative to the V_{max} with (6*R,S*)-methyl-PH_4 with phosphorylated tyrosine hydroxylase, which is defined as 1. [f] Calculated by eq 1. [g] The kinetic parameters of unphosphorylated tyrosine hydroxylase with (6*S*)-BH_4 cannot be quantitated (see text).

is important not only for substrate inhibition but also for producing a low K_m for tyrosine. The current results indicate that the chirality and length of the hydrocarbon chain also affect the binding of tyrosine, especially with phosphorylated enzyme [compare (6*S*)-methyl- with (6*S*)-propyl-PH_4, Table I]. Phosphorylation of tyrosine hydroxylase by cAMP-dependent protein kinase appeared to cause a shift to lower K_m for tyrosine with (6*S*)-propyl-PH_4 but had only a mild effect on tyrosine K_m with the unnatural (6*R*)-propyl-PH_4 as cofactor (Table I). The exact quantitation of K_m and V_{max} in cases of substrate inhibition, requires a knowledge of the mechanism. The ranges of values shown in Table I for (6*S*)-propyl-PH_4 and (6*R*)-BH_4 reflect analysis of the curves in Figure 3 by a variety of equations describing different types of substrate inhibition (Segel, 1975).

V_{max} and K_m for Cofactor. Hyperbolic cofactor K_m curves were produced by unphosphorylated and phosphorylated tyrosine hydroxylase with both the (6*R*) and (6*S*) isomers of all cofactors studied (Table II). With unphosphorylated tyrosine hydroxylase the K_m's for (6*R*)- and (6*S*)-propyl-PH_4 were identical to each other and an order of magnitude lower than the apparent K_m for (6*R*)-BH_4. However, (6*R*)-propyl-PH_4, which has the unnatural configuration at the 6-position, showed a 6-fold higher V_{max} than (6*S*)-propyl-PH_4. Despite the equality of their individual affinities, a 1:1 mixture of the (6*R*) and (6*S*) isomers of 6-propyl-PH_4 gave a V_{max} with unphosphorylated hydroxylase that was considerably slower than the average behavior predicted from eq 1 (see Discussion), though still somewhat faster than the pure natural isomer.

The apparent K_m for the unnatural (*R*) isomer of 6-propyl-PH_4 dropped by only 100-fold upon phosphorylation of tyrosine hydroxylase with cAMP-dependent protein kinase, whereas that of the natural (*S*) isomer decreased 2000-fold. The K_m of (6*S*)-propyl-PH_4 is the lowest yet reported for any cofactor, being 10 times below that of the natural cofactor (6*R*)-BH_4 and almost 20 times below that of the unnatural 6-propyl enantiomer. This is balanced by a 10-fold difference in maximum velocity between (6*R*)- and (6*S*)-propyl-PH_4, such that V_{max}/K_m for the natural isomer with phosphorylated hydroxylase is only 1.6-fold higher than that of the unnatural isomer (Table II, Figure 4). In comparing the rates of the two isomers, it should be noted that these reactions were performed at 200 μM tyrosine. This concentration is about 80% of saturation with the (6*R*) isomer but gives about 20% substrate inhibition with the natural (6*S*) isomer. The kinetics of phosphorylated tyrosine hydroxylase with two different mixtures are within experimental error of the values predicted by eq 1 for competing substrates. These large differences observed between the (6*R*) and (6*S*) isomers of 6-propyl-PH_4 are in contrast to (6*R*)- and (6*S*)-BH_4 which have similar

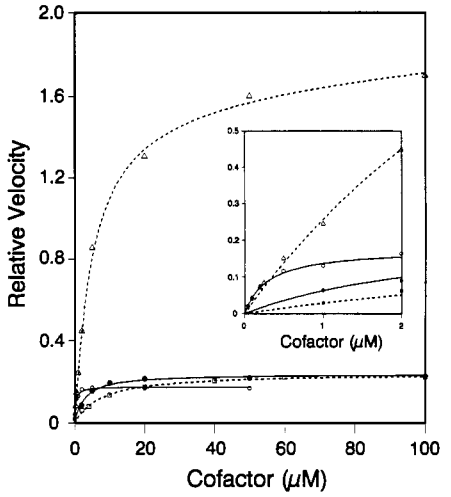

FIGURE 4: The rate of L-DOPA formation by tyrosine hydroxylase phosphorylated with cAMP-dependent protein kinase with varied cofactor: (6*R*)-BH_4 (●) and (6*S*)-BH_4 (□) (both at 100 μM tyrosine); (6*S*)-propyl-PH_4 (○) and (6*R*)-propyl-PH_4 (△) (both at 200 μM tyrosine); all reactions were at 37 °C and in 0.1 M KPO_4, pH 7.15. Solid and dashed lines (natural and unnatural C6 configurations, respectively) are best nonlinear least-squares fits to the Michaelis–Menten equation. All rates are relative to V_{max} of phosphorylated tyrosine hydroxylase with (6*R*,*S*)-methyl-PH_4, which is defined as 1 (tyrosine = 200 μM, (6*R*,*S*)-methyl-PH_4 varied). The inset is an expansion of the figure from 0 to 2 μM cofactor.

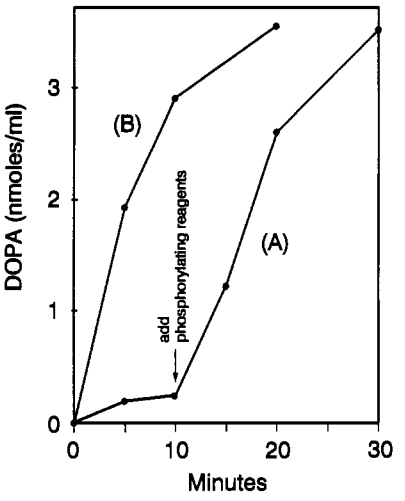

FIGURE 5: Effect of adding phosphorylating reagents to an ongoing reaction of unphosphorylated tyrosine hydroxylase with 1.0 mM (6*S*)-BH_4. (A) A mixture of 10 μmol of potassium phosphate (final pH 7.15 after addition of all components), 2 μmol of sodium ascorbate, 0.5 nmol of (*m*-hydroxybenzyl)hydrazine, 25 units of superoxide dismutase, 250 units of catalase, 0.1 μmol of (6*S*)-BH_4, and bovine striatal tyrosine hydroxylase were preincubated for 10 min at 37 °C, and the reaction was initiated with 20 nmol of tyrosine to give a final volume of 75 μL. After 10 min of reaction (arrow), 0.75 nmol unit of protein kinase A, 0.5 nmol of cAMP, and 0.5 μmol of $MgCl_2$ (all in 15 μL) and then 0.12 μmol of ATP (in 10 μL) were added to initiate phosphorylation. Samples were withdrawn at the indicated times and analyzed for DOPA as described under Materials and Methods. (B) Same as (A), but with all of the reaction components including the phosphorylating reagents (excluding cofactor) preincubated for 10 min at 37 °C and the reaction was initiated with 0.1 μmol of (6*S*)-BH_4 to give a total volume of 0.1 mL.

maximum velocities and affinities which differ by less than 3-fold (Figure 4, Table II). However, the reaction of (6*R*)-BH_4 is considerably inhibited by the 100 μM tyrosine used in this comparison (see Figure 3). A 2:1 (6*R*)/(6*S*) mixture of BH_4, typical of material produced by catalytic reduction in acid (Bailey & Ayling, 1978a), gave kinetic parameters very close to those of the pure natural isomer alone (Table II).

The unnatural (6*R*) isomer of 6-methyl-PH_4 was found to have over a 3-fold faster maximum velocity with phosphorylated tyrosine hydroxylase than the 1:1 (6*R*,*S*)-methyl-PH_4 mixture, the fastest rate so far observed for any cofactor with tyrosine hydroxylase (Table II). This is compensated for by an unusually high K_m. Equation 1 predicts that pure (6*S*)-methyl would have a K_m about two-thirds that of the racemic mix and a $V_{max(R)}/V_{max(S)}$ ratio of 11, a value similar to that with the enantiomers of the 6-propyl analogue.

Reaction of (6S)-BH_4 with Unphosphorylated Tyrosine Hydroxylase. Although progress curves of phosphorylated tyrosine hydroxylase with (6*S*)-BH_4 as cofactor are similar to those with other cofactors, the progress of DOPA formation by unphosphorylated tyrosine hydroxylase with (6*S*)-BH_4 proved to be so anomalous that definitive kinetic constants could not be calculated. At all concentrations of cofactor examined (0.5–3 mM) an initial burst of DOPA was produced after initiation with tyrosine. By 10 min a slower rate was established which remained linear up to at least 30 min (Figure 2). At all times during these reactions the amount of DOPA formed increased with increasing (6*S*)-BH_4 in a manner suggesting a process saturable only at a concentration higher than 3 mM cofactor. At 3 mM (6*S*)-BH_4 the slower final rate was about 1.5% that of V_{max} for (6*R*)-BH_4 with unphosphorylated hydroxylase when assayed with 0.1 mM tyrosine. The initial rate obtained by fitting progress curves of DOPA formation with 3 mM (6*S*)-BH_4 to an equation for exponential decay of a starting rate to a final steady rate was estimated to be between 10 and 15% of the V_{max} for (6*R*)-BH_4. Control experiments, in which NADH/DHPR was used instead of sodium ascorbate gave similar results. If enzyme is not preincubated with cofactor for 10 min, a slow linear rate is observed which is equal to the final rate of the preincubated reactions (Figure 2).

To examine the nature of the declining rate, a standard (6*S*)-BH_4 reaction with unphosphorylated tyrosine hydroxylase was initiated with tyrosine. After 10 min all of the components of the standard phosphorylating system (protein kinase, cAMP, $MgCl_2$, and finally ATP) were added and the reaction continued for a further 20 min. The progress curves (Figure 5) show that after addition of phosphorylation reagents product formation accelerates almost to the same rate as in a reaction of (6*S*)-BH_4 with tyrosine hydroxylase which has been phosphorylated initially. The inactivation that occurs with (6*S*)-BH_4 by 10 min with unphosphorylated hydroxylase is therefore either reversed or made ineffective by phosphorylation. Similar results were obtained at 0.1 and 1 mM (6*S*)-BH_4.

Phenylalanine Hydroxylase

Progress Curves. Phenylalanine hydroxylase requires activation by phenylalanine for maximum activity which is complete in 2–3 min at 27 °C (Ayling & Helfand, 1976; Shiman, 1985). Although the time course of activation is unchanged in the presence of 6-methyl-PH_4, it is strongly inhibited by (6*R*)- and (6*S*)-BH_4 (Bailey & Ayling, 1978). Investigation of the dependence of the inhibition on the structure of the cofactor revealed that neither (6*R*)- nor (6*S*)-propyl-PH_4 inhibits the activation. Thus, the inhibition appears to be specific to one or both of the hydroxyl groups of the side chain. However, even after preincubation with phenylalanine, purified phenylalanine hydroxylase displayed a short lag with all of the cofactors in Table III in the spectral assay but not in the tyrosine assay. This lag was found to be due to an accumulation of C4a-hydroxydihydropterin, since

Table III: Kinetic Constants for the C6 Enantiomers of 6-Propyl-PH_4, 6-Methyl-PH_4, and BH_4 with Phenylalanine Hydroxylase

	K_m for phenylalanine[a]		K_m for cofactor[a]		
cofactor/isomer	K_m (mM)	V_{max}[c]	K_m (mM)	V_{max}[c]	V_{max}/K_m
6-propyl-PH_4[b]					
(*S*)	0.13[e]	0.22	0.002	0.2	100
(*R*)	0.25	1.5	0.006	1.2	200
1:1 (*R*)/(*S*)			0.003	0.35	120
1:1 (*R*)/(*S*) calcd[g]			0.003	0.45	150
6-methyl-PH_4[b]					
(*R*)	0.25	1.25	0.068	1.3	19
1:1 (*R*)/(*S*)	0.3	1.0	0.062	1	16
(*S*) calcd[g]			0.057	0.7	13
BH_4[d]					
(*R*)	0.17[e,f]		0.021	1	48
(*S*)	0.17[f]		0.025	0.27	11

[a] The apparent K_m's for phenylalanine (measured over a range of up to 1.0 and 2.0 mM phenylalanine for 6-propyl-PH_4 and 6-methyl-PH_4, respectively) were determined at 0.2 mM cofactor, except (6*S*)-propyl-PH_4 which was at 0.05 mM. Apparent K_m's for cofactor (measured over a range of up to 50 and 500 μM for 6-propyl-PH_4 and 6-methyl-PH_4, respectively) were determined at 1.0 mM phenylalanine. [b] Both (6*S*)-propyl-PH_4 and (6*S*)-methyl-PH_4 have the same C6 chirality as the natural isomer of tetrahydrobiopterin, (6*R*)-BH_4. [c] Apparent maximum velocities are relative to the apparent V_{max} with (6*R*,*S*)-methyl-PH_4 at 1.0 mM phenylalanine, which is defined as 1. [d] Kinetic constants from Bailey and Ayling (1978a). [e] Substrate inhibited above 1 mM phenylalanine. [f] K_m for a sigmoidal saturation curve. [g] Calculated by eq 1.

it was entirely eliminated by inclusion of 10 μg of C4a-carbinolamine dehydratase in the reaction. It is of interest to note that this effect with both enantiomers of 6-propyl-PH_4 extends the range of known substrates for the dehydratase (Lazarus et al., 1983).

K_m for Phenylalanine. The apparent K_m for phenylalanine in the presence of nearly saturating ($\sim 25K_m$) 6-propyl-PH_4 was found to be about 2 times higher with the unnatural (6*R*)- than with (6*S*)-propyl-PH_4 (Table III). With a 6-methyl substituent, chirality at the 6-position has little effect on the apparent K_m for phenylalanine. Saturation curves for phenylalanine (up to 10 mM) with (6*R*)-propyl-PH_4 and (6*R*)- and (6*R*,*S*)-methyl-PH_4 were hyperbolic, in contrast to the sigmoidal curve observed when BH_4 is the cofactor (Ayling & Helfand, 1976; Bailey & Ayling, 1978a). A nearly hyperbolic curve was also seen with (6*S*)-propyl-PH_4 at phenylalanine less than 0.7 mM. However, as the concentration of phenylalanine is increased above 1 mM, the rate decreases. This relationship of substrate inhibition with the natural (6*S*)-propyl-PH_4 but not with (6*R*)-propyl-PH_4 is similar to that reported for (6*R*)-BH_4 vs (6*S*)-BH_4 as cofactor (Bailey & Ayling, 1978a).

V_{max} and K_m for Cofactor. As with all tetrahydrobiopterin analogues so far studied, hyperbolic saturation curves for cofactor were observed. The kinetic constants are summarized in Table III. The apparent K_m (at 1 mM phenylalanine) for natural (6*S*)-propyl-PH_4 was 3-fold lower than for the (6*R*) isomer. The V_{max} of the natural isomer was also 6-fold slower. Thus, V_{max}/K_m for the natural (6*S*) isomer is only 2-fold lower than for (6*R*)-propyl-PH_4. In contrast to the propyl group, the unnatural (*R*) isomer of 6-methyl-PH_4 gave only slight increases in K_m and V_{max} over the racemic (6*R*,*S*)-methyl-PH_4. Calculation by eq 1 suggests that the ratio $V_{max(R)}/V_{max(S)}$ may only be about 2 and that the K_m for the unnatural 6-methyl configuration may only be 20% higher than the natural configuration. Phenylalanine hydroxylase reactions with (6*R*)- and (6*S*)-propyl-PH_4 and (6*R*)-methyl-PH_4, as with (6*R*,*S*)-methyl-PH_4 and (6*R*)- and (6*S*)-BH_4, were all found to be closely coupled, i.e., a mole of tyrosine produced for each mole of cofactor consumed.

Discussion

Cofactor Chirality and Substrate Inhibition of Tyrosine Hydroxylase by Tyrosine. Inhibition of tyrosine hydroxylase by tyrosine has appeared until now to be associated exclusively with the presence of 1′- and/or 2′-hydroxyl groups in the cofactor 6-substituent. No purely hydrocarbon 6-substituent produced any such response, including (6*R*,*S*)-ethyl-PH_4 (Bailey et al., 1989). The current results reveal a mild substrate inhibition in the presence of (6*S*)-propyl-PH_4 with both phosphorylated and unphosphorylated enzyme (Figure 3).

Substrate inhibition of unphosphorylated tyrosine hydroxylase has been observed previously with a number of 6-(hydroxyalkyl)tetrahydropterins in assays performed at pH 6. The extent of inhibition by tyrosine with (6*R*,*S*)-D-*threo*-BH_4 as cofactor was similar to that with (6*R*,*S*)-L-*erythro*-BH_4 (both having the same chirality of the 1′-hydroxyl group). However, the two diastereoisomers having the opposite 1′-OH chirality, (6*R*,*S*)-L-*threo*- and (6*R*,*S*)-D-*erythro*-BH_4, showed nearly hyperbolic responses to increasing tyrosine (Numata et al., 1977). Similarly, (6*R*,*S*)-L-1′-hydroxyethyl, but not the D isomer, promoted mild substrate inhibition (Kato et al., 1980b). The findings that the (6*S*)-propyl group alone (Figure 3), the short-chain (6*R*,*S*)-hydroxymethyl (Bailey et al., 1989), and even (6*R*,*S*)-L-1′-hydroxyethyl (Kato et al., 1980b) produce only mild inhibition in comparison to that found with (6*R*)-BH_4 suggest a synergism between a 1′-hydroxyl group with the correct configuration and the full propyl hydrocarbon chain.

Substrate inhibition by tyrosine is not induced by (6*S*)-BH_4 even though it possesses a side chain identical to that of (6*R*)-BH_4, which can also project equatorially from the pyrazine ring. A structural basis for this observation has been examined with the aid of molecular mechanics. Regardless of which is the preferred bound conformation of (6*S*)-BH_4, none has both the 1′-hydroxyl and 3′-methyl groups projecting into the same positions in space relative to the pyrimidine ring as in any of the stable (6*R*)-BH_4 conformers (Ayling et al., 1990a). However, efficient utilization of the cofactor likely requires a specific coordination of the pyrimidine moiety with the catalytic site (Kaufman & Fisher, 1974; Bailey & Ayling, 1978b). Thus, despite the presence of an L-*erythro*-1′-hydroxyl group, (6*S*)-BH_4 may not be able to interact with the hydroxylase residues responsible for substrate inhibition by tyrosine.

Cofactor Chirality and Interaction of Amino Acid Substrate with Phenylalanine Hydroxylase. Earlier studies have shown that the response to varied phenylalanine is sigmoidal with tetrahydrobiopterin as cofactor due to the existence of an allosteric site for phenylalanine separate from the catalytic site (Ayling & Helfand, 1976; Bailey & Ayling, 1978a; Shiman, 1985). With (6*R*,*S*)-methyl-PH_4 as cofactor, phe-

nylalanine saturation curves are hyperbolic (Phillips et al., 1984). The nearly hyperbolic Michaelis–Menten curves for phenylalanine (below 1 mM) using (6*S*)-propyl-PH_4 as cofactor found in the current results further specify that this property of BH_4 is particularly dependent on one or both of the side-chain hydroxyl groups. On the other hand, a propyl group with the natural configuration appears to be sufficient to elicit inhibition by high phenylalanine similar to that observed with (6*R*)-BH_4. This suggests different roles for the cofactor 1′- and 2′-hydroxyls with phenylalanine and tyrosine hydroxylases. With the latter, especially when phosphorylated, these are required to promote maximal inhibition by tyrosine (see above) in the physiological range of this substrate.

Effect of Chirality on Cofactor Kinetics with Phenylalanine Hydroxylase and Phosphorylated Tyrosine Hydroxylase. The K_m's for 6-propyl-PH_4 with both phenylalanine hydroxylase and phosphorylated tyrosine hydroxylase are significantly influenced by side-chain chirality (Table II). The K_m's for the natural (6*S*) isomer with these two enzymes are 3 and 17 times lower, respectively, than for the unnatural isomer. These differences are counterbalanced by opposing changes in maximum velocity such that V_{max}/K_m is nearly maintained. A possible explanation for this observation is that relative to (6*S*)-propyl-PH_4 the unnatural isomer may destabilize initial cofactor interactions with the enzyme–substrate complex, but in a manner that is mostly unrecognized in the transition state of the rate-limiting step (Fersht, 1985).

The kinetics of phenylalanine hydroxylase and phosphorylated tyrosine hydroxylase with mixtures of (6*R*) and (6*S*) enantiomers agree well with those predicted by a simple equation for the response to two alternate substrates. In those cases where the K_i for two competing substrates (in this case cofactor isomers) equals their respective K_m's, the total velocity for the formation of a common product (e.g., hydroxylated amino acid) due to a mixture of both is usually expressed as

$$V_{total} = \frac{V_{max(A)}\frac{[A]}{K_{m(A)}} + V_{max(B)}\frac{[B]}{K_{m(B)}}}{1 + \frac{[A]}{K_{m(A)}} + \frac{[B]}{K_{m(B)}}} \quad (1a)$$

where $V_{max(A)}$, $V_{max(B)}$, $K_{m(A)}$, $K_{m(B)}$ are the kinetic constants of the separate species (Segel, 1975). The above equation can be rearranged into the form of a new Michaelis–Menten equation: $v_{total} = SV_{max(A+B)}/(S + K_{M(A+B)})$ where

$$V_{max(A+B)} = \frac{rV_{max(A)}K_{m(B)} + V_{max(B)}K_{m(A)}}{rK_{m(B)} + K_{m(A)}}$$

$$K_{m(A+B)} = \frac{(1 + r)K_{m(A)}K_{m(B)}}{rK_{m(B)} + K_{m(A)}} \quad (1b)$$

$r = [A]/[B]$, and $V_{max(A+B)}$ and $K_{m(A+B)}$ are the kinetic constants for the total concentration of both species. A consequence of this equation is that if a large difference exists between the K_m's for the alternate substrates, V_{max} and K_m of an equal mixture of the two will be dominated by the characteristics of the higher affinity component. This can be seen with both phenylalanine hydroxylase and phosphorylated tyrosine hydroxylase where racemic mixtures give kinetic parameters closer to those of the natural isomer for both phenylalanine hydroxylase and phosphorylated tyrosine hydroxylase.

The effect of side-chain chirality can be viewed in the context of changes in enzyme kinetic parameters due to alteration of the size of the 6-substituent. Within either class of enantiomers, increasing the length from 6-methyl to 6-propyl with both phenylalanine hydroxylase and phosphorylated tyrosine hydroxylase results in a considerable decrease in K_m for the cofactor. For example, with the latter enzyme there is a 2 order of magnitude difference in K_m between (6*S*)-methyl- (calculated) and (6*S*)-propyl-PH_4 yet only a 2-fold decrease in V_{max}. This phenomenon has been observed to a varying extent with a number of racemic hydrocarbon 6-substituents with both phenylalanine and tyrosine hydroxylase (Ayling & Bailey, 1983; Levine et al., 1987; Armarego et al., 1987; Reinhard et al., 1988; Bailey et al., 1989). The current work suggests that these earlier comparisons may primarily reflect the kinetics of the natural enantiomer.

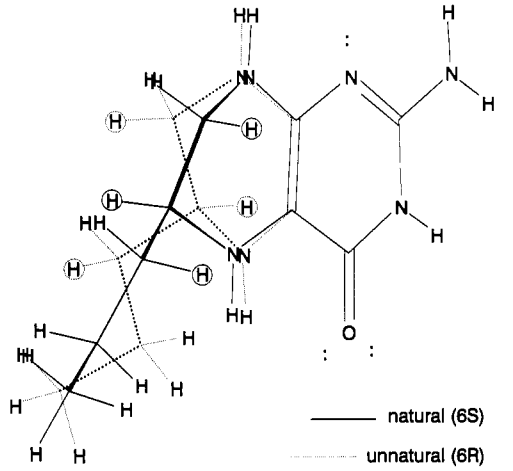

FIGURE 6: The global minimum energy conformers of (6*R*)- and (6*S*)-propyl-PH_4, superimposed with optimal overlap of only the atoms of the pyrimidine ring. Circled pairs of hydrogens (H_6, H_{7ax} and $H_{1'}$) mark the three regions where the two structures are most divergent, and therefore may represent likely sites for differential recognition of C6 chirality by phenylalanine and tyrosine hydroxylases.

Phenylalanine hydroxylase is less sensitive to the chirality of 6-propyl-PH_4 than phosphorylated tyrosine hydroxylase. It also appears to be more dependent on a longer 6-substituent for expression of stereoisomer recognition. This suggests that a somewhat different set of atoms may be responsible for the destabilization of the initial enzyme/cofactor complex by the unnatural enantiomer. Proton NMR coupling constants (Weber & Viscontini, 1975; Armarego et al., 1984; Williams & Storm, 1985) and X-ray crystal diffraction data (Matsuura et al., 1985a) have shown that the 6-substituents of otherwise underivatized tetrahydropterins are primarily pseudoequatorial to the pyrazine ring. With the side chain in the equatorial conformation, the axial C6 and C7 hydrogens in 6-position enantiomers project to opposite sides of the pyrazine ring (Figure 6). Further, even with (6*R*)-methyl-PH_4, the three equivalent rotamers of this simplest side chain still place a methyl hydrogen in a position where none would normally be located. Three hydrogens , H_6, H_{7ax}, and one $H_{1'}$, appear to be primary candidates for any steric hindrance to interaction of either (6*R*)-methyl-PH_4 or (6*R*)-propyl-PH_4 with phosphorylated tyrosine hydroxylase. However, of these, H_6 seems least likely, since 6,6-dimethyl-PH_4 has a similar K_m and V_{max} (Bailey et al., 1989) to the calculated values for (6*S*)-methyl-PH_4. With phenylalanine hydroxylase, which only weakly differentiates between (6*R*)- and (6*S*)-methyl-PH_4, chirality-induced destabilization of (6*R*)-propyl-PH_4 interactions may be due to propagation of a preferred conformation of C1′ to staggered conformations of C2′ and/or C3′. Alternatively, the hydrophobic interactions of the longer (6*R*)-propyl side chain with phenylalanine hydroxylase may

freeze conformations of the pyrazine ring and/or C1′ so that H_{7ax}, H_6, or $H_{1'}$ are more effectively destabilizing.

Comparison of the natural (*S*) isomer of 6-propyl-PH_4 with (6*R*)-BH_4 reveals the impact of the presence of only the 1′- and 2′-hydroxyl groups. The K_m of (6*R*)-BH_4 for phosphorylated tyrosine hydroxylase is 10-fold higher than that of the simpler propyl analogue. However, the V_{max} of (6*R*)-BH_4, which at optimal tyrosine concentration is about double the value listed in Table II, is minimally 3 times higher than that of (6*S*)-propyl-PH_4. Therefore, one or both of the hydroxyls in the natural cofactor may destabilize the complex with phosphorylated tyrosine hydroxylase. The data of Kato et al. (1980) with unphosphorylated bovine adrenal tyrosine hydroxylase assayed at pH 6 are, in part, consistent with this effect, since (6*R*,*S*)-L-(1′,2′-dihydroxyethyl)-PH_4 was found to have a higher K_m and somewhat higher V_{max} than the simpler (6*R*,*S*)-L-(1′-hydroxyethyl)-PH_4. A similar effect can be seen with phenylalanine-activated phenylalanine hydroxylase (Table III).

Kinetics of Unphosphorylated Tyrosine Hydroxylase. Investigations of whether phosphorylation by cAMP-dependent protein kinase changes only K_m or also V_{max} have lead to differing conclusions (Kaufman & Kaufman, 1985). Although part of the problem has been the variation of hydroxylase kinetics with pH, the current results at pH 7.15 show a second factor: the structure of the cofactor. For example, with the unnatural (6*R*)-propyl-PH_4, unphosphorylated and phosphorylated tyrosine hydroxylase both give hyperbolic tyrosine saturation curves with the same V_{max}. With (6*R*)-BH_4, taking into account the extremely high K_m of this cofactor with unphosphorylated enzyme, and with measurements taken at the optimal tyrosine concentration, similar rates are also observed. Furthermore, there is only a small decrease in maximum measured rate with (6*S*)-propyl-PH_4 upon phosphorylation, which may be accounted for by the greater substrate inhibition by tyrosine in this state. However, at neutral pH the maximum velocity of DOPA formation with a few analogues such as (6*R*,*S*)-methyl-PH_4 and 6,6-dimethyl-PH_4 (Bailey et al., 1989) increases 3–4-fold upon phosphorylation. Comparison of (6*R*,*S*)-propyl-PH_4 with (6*R*,*S*)-ethyl-PH_4 (Bailey et al., 1989) and (6*R*,*S*)-methyl-PH_4 shows that the main impact of decreasing side-chain length with unphosphorylated tyrosine hydroxylase is to increase apparent K_m for cofactor (5-fold overall), with only a small (about 40%) decrease in maximum velocity. The stimulation of V_{max} upon phosphorylation that is associated primarily with (6*R*,*S*)-methyl-PH_4 hints that at neutral pH this cofactor may not support a fully coupled reaction with the unphosphorylated enzyme. It has been reported that DOPA is produced by unphosphorylated tyrosine hydroxylase at the same rate that (6*R*,*S*)-methyl-PH_4 is consumed (Shiman et al., 1971). However, these earlier experiments were performed at pH 6, a condition that appears to mimic the decreased K_m for cofactor caused by phosphorylation.

The extremely low and decelerating rates of DOPA formation by unphosphorylated enzyme in the presence of (6*S*)-BH_4 are not due to a fast irreversible inactivation. This can be seen from the acquisition of nearly the full rate expected of phosphorylated hydroxylase upon adding the reagents necessary for cAMP-dependent protein phosphorylation to a reaction of unphosphorylated enzyme 10 min after initiation (Figure 5). The deceleration observed appears unrelated to the reported inactivation of unphosphorylated tyrosine hydroxylase purified from PC12 cells, in which (6*R*)-BH_4 was more potent than (6*S*)-BH_4 in incubations without turnover (Kuhn & Lovenberg, 1983). The possibility that with unphosphorylated tyrosine hydroxylase (6*S*)-BH_4 may induce a partial oxidation of the enzyme bound iron to the ferric state during turnover has been proposed and is discussed in more detail elsewhere (Ayling et al., 1990a).

Conclusion. Though the function of the 1-*erythro*-dihydroxypropyl group in (6*R*)-BH_4 with phenylalanine hydroxylase appears to be different from that with tyrosine hydroxylase, its mechanism of action is similar in both. With the latter enzyme neither the sigmoidal K_m curves nor activation by substrate characteristic of the former has yet been detected. Instead, the cofactor side chain enables a strong substrate inhibition by tyrosine. These differences are not unexpected since the role of tyrosine hydroxylase is to control the level of the ultimate catecholamine product while that of phenylalanine hydroxylase is to maintain the level of the substrate. However, comparison of (6*S*)-propyl-PH_4 with (6*R*)-BH_4 suggests that the hydroxyl groups are destabilizing with both hydroxylases. Therefore, the hydrocarbon chain may supply a platform of enzyme affinity against which the hydroxyls can exert their interactive forces, which in turn promote enzymic regulation rather than decrease K_m for cofactor.

There are several practical applications of the pure isomers. The extremely high maximum velocity of phenylalanine and unphosphorylated tyrosine hydroxylases with (6*R*)-propyl-PH_4 makes it a valuable tool for detection of very low levels of hydroxylase activity. The natural (6*S*) isomer of propyl-PH_4, on the other hand, possesses the highest V_{max}/K_m yet found for a cofactor of tyrosine hydroxylase phosphorylated by cAMP-dependent protein kinase and therefore may find use in replacement therapy for (6*R*)-BH_4-deficiency states.

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