# Changes in the Cofactor Binding Domain of Bovine Striatal Tyrosine Hydroxylase at Physiological pH upon cAMP-Dependent Phosphorylation Mapped with Tetrahydrobiopterin Analogues†

Steven W. Bailey, Shirley B. Dillard, K. Bradford Thomas, and June E. Ayling*
*Department of Pharmacology, College of Medicine, University of South Alabama, Mobile, Alabama 36688*
*Received June 16, 1988; Revised Manuscript Received September 14, 1988*

ABSTRACT: The structure of the cofactor binding domain of tyrosine hydroxylase (TH) was examined at physiological pH by determining kinetic parameters of (*R*)-tetrahydrobiopterin [(*R*)-$BH_4$] and a series of tetrahydropterin ($PH_4$) derivatives (6-$R_1$-6-$R_2$-$PH_4$: $R_1$ = H and $R_2$ = methyl, hydroxymethyl, ethyl, methoxymethyl, phenyl, and cyclohexyl; $R_1$ = methyl and $R_2$ = methyl, ethyl, propyl, phenyl, and benzyl). A minimally purified TH preparation that was not specifically phosphorylated (designated as "unphosphorylated") was compared with enzyme phosphorylated with cAMP-dependent protein kinase. The $K_m$ for tyrosine with most tetrahydropterin analogues ranged between 20 and 60 $\mu$M with little decrease upon phosphorylation. Two exceptions were an unusually low $K_m$ of 7 $\mu$M with 6-ethyl-$PH_4$ and a high $K_m$ of 120 $\mu$M with 6-phenyl-6-methyl-$PH_4$, both with phosphorylated TH. Tyrosine substrate inhibition was elicited only with (*R*)-$BH_4$ and 6-hydroxymethyl-$PH_4$. With unphosphorylated TH (with the exception of 6-benzyl-6-methyl-$PH_4$, $K_m$ = 4 mM) an inverse correlation between cofactor $K_m$ and side-chain hydrophobicity was observed ranging from a high with (*R*)-$BH_4$ (5 mM) to a low with 6-cyclohexyl-$PH_4$ (0.3 mM). An 8-fold span of $V_{max}$ was seen overall. Phosphorylation caused a 0.6–4-fold increase in $V_{max}$ and a 35–2000-fold decrease in $K_m$ for cofactor, ranging from a high of 60 $\mu$M with 6-methyl-$PH_4$ to a low of 0.6 $\mu$M with 6-cyclohexyl-$PH_4$. A correlation of the size of the hydrocarbon component of the side chain with affinity is strongly evident with phosphorylated TH, but in contrast to unphosphorylated enzyme, the hydroxyl groups in hydroxymethyl-$PH_4$ (20 $\mu$M) and (*R*)-$BH_4$ (3 $\mu$M) decrease $K_m$ in comparison to that of 6-methyl-$PH_4$. Although 6,6-disubstituted analogues were found with affinities near that of (*R*)-$BH_4$ (e.g., 6-propyl-6-methyl-$PH_4$, 4 $\mu$M), they were frequently more loosely associated with phosphorylated TH than their monosubstituted counterparts (6-phenyl-$PH_4$, 0.8 $\mu$M; cf. 6-phenyl-6-methyl-$PH_4$, 8 $\mu$M). A model of the cofactor side-chain binding domain is proposed in which a limited region of nonpolar protein residue(s) capable of van der Waals contact with the hydrocarbon backbone of the (*R*)-$BH_4$ dihydroxypropyl group is opposite to a recognition site for hydroxyl(s). Although interaction with either the hydrophilic or hydrophobic regions of unphosphorylated tyrosine hydroxylase is possible, phosphorylation by cAMP-dependent protein kinase appears to optimize the simultaneous operation of both forces.

Little is known about the catalytic site of tyrosine hydroxylase, the first enzyme in the biosynthetic pathway of the catecholamines, especially in relation to the forces that govern the binding of the cofactor (*R*)-tetrahydrobiopterin [(*R*)-$BH_4$][1] (Figure 1). Currently, it appears that the minimal requirement for cofactor activity is the basic tetrahydropterin ring (Kaufman & Kaufman, 1985). Considering the overall task of cofactor as a reducing agent, it is not surprising that both electron-donating groups, at positions 2 and 4 (amino and hydroxyl), are required (Ellenbogen et al., 1965). On the other hand, although the kinetic parameters of tyrosine hydroxylase have been observed to depend significantly on the 6-substituent, a considerable variation of structure of this group is tolerated. In contrast to phenylalanine hydroxylase, Shiman et al. (1971) have shown that simple tetrahydropterin produces an almost completely coupled hydroxylation yielding 0.87 mol of DOPA per mole of cofactor oxidized. Numata et al. (1977) and Kato et al. (1980) have shown that at pH 6 several properties of the tyrosine hydroxylase reaction depend on the presence and stereochemistry of the 1′- and 2′-hydroxyl groups. Recently, Bigham et al. (1987) and Armarego et al. (1987) have reported studies of the effect of alkoxy and alkyl substituents, also at pH 6. However, to date, no comprehensive study of the function of the pteridine 6-substituent at physiological pH has been reported. Major alterations in the kinetics of both tetrahydrobiopterin and 6-methyltetrahydropterin have been observed in reactions performed near pH 7 as compared to more acidic environments (Markey et al., 1980; Pollock et al., 1981; Pradhan et al., 1981; Lazar et al., 1982; Miller & Lovenberg, 1985).

Tyrosine hydroxylase can be phosphorylated in vitro by the action of $Ca^{2+}$/calmodulin, $Ca^{2+}$/phospholipid, and cAMP-dependent protein kinases (Kaufman, 1987). The interplay among these systems in vivo is only slowly being revealed and is a primary concern for understanding the regulation of catecholamine production. Of the different phosphorylating

† This investigation was supported by Grant GM-30368 awarded by the National Institute of General Medical Sciences, Grant CA 31852 awarded by the National Cancer Institute, Contract NS 4-2368 from the National Institute of Neurological and Communicative Disorders and Stroke, and a Grant-in-Aid from Ciba Geigy. Mass spectra were performed at the University of South Alabama Mass Spectrometry Center, supported by NIH Grant RR 02973. Preliminary reports of part of this work have been presented (Ayling & Bailey, 1984; Ayling et al., 1986).

[1] Abbreviations: $BH_4$, tetrahydrobiopterin [6-(1-*erythro*-dihydroxypropyl)tetrahydropterin]; (*R*)-$BH_4$, 6*R* diastereoisomer of $BH_4$ (the natural isomer); protein kinase A, cAMP-dependent protein kinase; $PH_4$, tetrahydropterin; DOPA, dihydroxyphenylalanine; HPLC, high-pressure liquid chromatography; $Na_2EDTA$, ethylenediaminetetraacetic acid disodium salt; FAB, fast atom bombardment.

0006-2960/89/0428-0494$01.50/0 

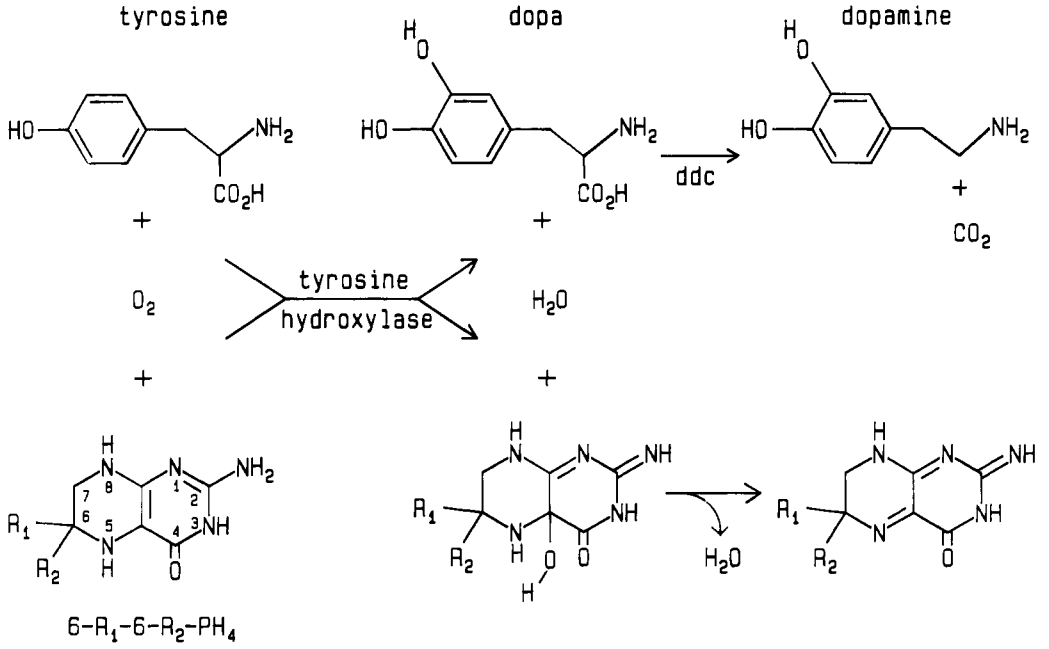


FIGURE 1: Reaction of tyrosine hydroxylase. The natural cofactor is tetrahydrobiopterin: $R_1$ = 1′,2′-dihydroxypropyl; $R_2$ = H. The DOPA produced is converted to dopamine by DOPA decarboxylase (ddc).

systems, cAMP-dependent protein kinase has been reported as having the greatest impact on the properties of tyrosine hydroxylase, mainly via a decrease in the $K_m$ for cofactor (Lloyd & Kaufman, 1975; Lovenberg et al., 1975; Yamauchi & Fujisawa, 1979; Markey et al., 1980; Vulliet et al., 1980; Pollock et al., 1981; Lazar et al., 1982; Miller & Lovenberg, 1985). The following experiments were designed to provide an indication of the nature of the interaction at physiological pH, between the cofactor binding domain of tyrosine hydroxylase and the substituent(s) at C6 (Figure 2), and to show how these change upon cAMP-dependent phosphorylation. The results suggest that a set of analogues may provide a probe for the state of phosphorylation in vivo.

## Materials and Methods

*Reagents.* Bovine erythrocyte superoxide dismutase and cAMP-dependent protein kinase from bovine heart were purchased from Sigma. Bovine liver catalase was from Worthington. Spherisorb ODS II, Partisil SCX, and Rsil SCX, stationary phases for HPLC, were obtained from Alltech Associates, and Bakerbond ODS was from J. T. Baker. Chelex 100 was purchased from Bio-Rad. (*R*)-Tetrahydrobiopterin, (*R,S*)-tetrahydrobiopterin, and (*R,S*)-6-methyltetrahydropterin were from Dr. B. Schircks, CH 8645 Jona, Switzerland. (*R,S*)-6-(Hydroxymethyl)- and (*R,S*)-6-(methoxymethyl)-tetrahydropterin were kindly donated by Dr. Wolfgang Pfleiderer. 6,6-Dimethyltetrahydropterin was synthesized as described previously (Bailey & Ayling, 1983). (*R,S*)-6-Benzyl-6-methyl-, (*R,S*)-6-phenyl-6-methyl-, (*R,S*)-6-propyl-6-methyl-, (*R,S*)-6-ethyl-6-methyl-, and (*R,S*)-6-ethyltetrahydropterin were synthesized either by a modification of this procedure or by an alternate synthetic route to be published elsewhere.[2] 6-Hydroxymethyl- and 6-methoxymethyl-$PH_4$ were purified by chromatography on Rsil-SCX (25 × 0.9 cm) eluted with ammonium acetate, pH 4.8 (0.1 M ammonia), or ammonium formate, pH 3.3 (0.07 M ammonia), respectively, at 4 mL/min. All tetrahydropterins were analyzed for purity by HPLC and were used in enzymatic reactions when greater than 99% of the electrochemical response at +0.3 V vs Ag/AgCl (Bioanalytical Systems dual glassy carbon electrode MF1000/MF1018), and 99% of the UV absorbance at 265 nm, resided in a single peak. Molar extinction coefficients of tetrahydropterins were determined by 2,6-dichlorophenolindophenol titration, with ascorbic acid as standard. The concentrations of tetrahydropterins and their state of reduction were checked from absorption spectra which were routinely run immediately before use in enzymatic reactions. Structural identity was confirmed by mass spectrometry. Fast atom (xenon) bombardment (FAB) spectra were obtained on a VG70-250SEQ mass spectrometer with glycerol/dimethyl sulfoxide (3:1) as solvent.

The hydrophobic character of each tetrahydropterin was assessed from its retention as the neutral species on a $C_{18}$ reversed-phase HPLC column (Figure 2). The partitioning between carbonaceous stationary phases and primarily aqueous mobile phases has been shown, within certain limitations, to correlate well with solute partitioning in other water/organic solvent systems (Horvath et al., 1977; Sadek et al., 1985; Tchapla et al., 1988). The 12 tetrahydropterins show increasing retention with increased hydrocarbon surface area. A lower capacity factor ($k'$) was found for 6-methoxymethyl- than for 6-ethyl-$PH_4$ as expected due to its interaction as a hydrogen-bond acceptor with the eluant. (*R*)-$BH_4$ displayed an anomalously high $k'$ compared to 6-hydroxymethyl-$PH_4$. This is likely due to unusually strong interactions of vicinal hydroxyls with free silanol groups on the stationary phase (Bailey & Ayling, 1980), since this excess retention largely disappeared at higher sample loads.

*Synthesis.* 6-Phenyl-7,8-dihydropterin was obtained by the method of Pfleiderer and Mengel (1971) modified primarily by the use of iron and acetic acid for higher yield in the reduction of 2-amino-5-nitro-6-(phenacylamino)-4(3*H*)-pyrimidinone. Although sodium dithionite is frequently used for such reductions, it was not very effective with this particular compound. The conditions of the catalytic hydrogenation of 6-phenyl-7,8-dihydropterin to 6-phenyl-5,6,7,8-tetrahydropterin were chosen to minimize overreduction. Further hydrogenation to 6-cyclohexyl-5,6,7,8-tetrahydropterin, on the other hand, was facilitated by the activity of platinum catalyst toward phenyl rings.

[2] J. E. Ayling, et al., unpublished results.

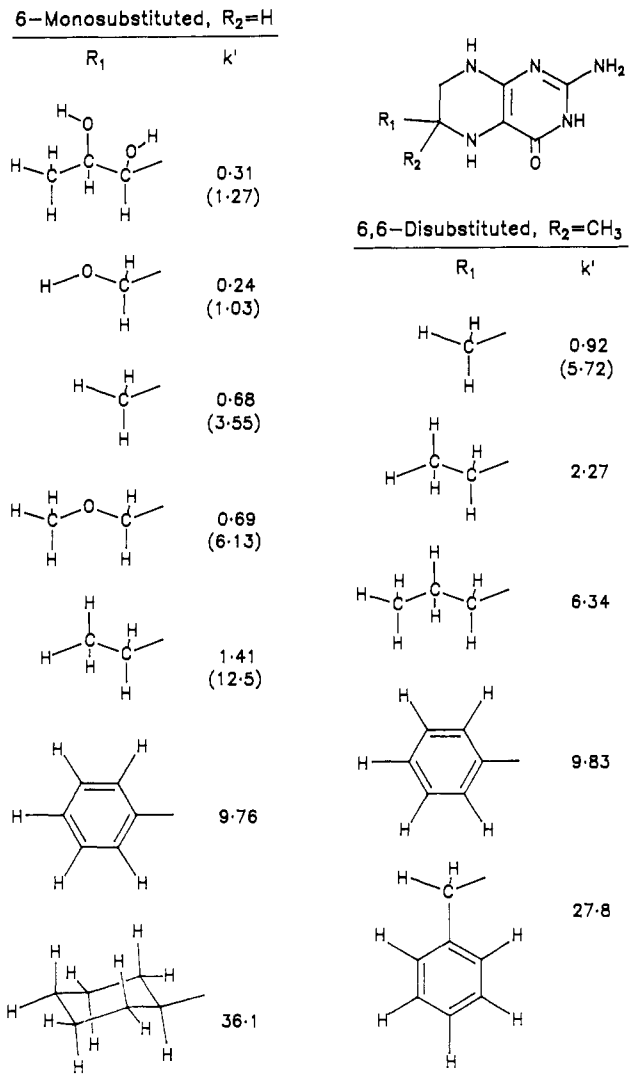


FIGURE 2: Structure of the 6-substituents ($R_1$ and $R_2$) used as probes of the cofactor binding domain of bovine striatal tyrosine hydroxylase and their retentions (expressed as capacity factors, $k'$) on reverse-phase HPLC, as an index of relative hydrophobicity. HPLC conditions: Spherisorb ODS II, 3 μm, 15 × 0.46 cm, eluted at 1 mL/min with potassium phosphate, pH 6.5 (0.05 M in potassium), containing 1 mM $Na_2EDTA$/methanol (9:1). Numbers in parentheses are for buffer/methanol (98:2).

(*1*) *6-Phenyl-7,8-dihydropterin.* A deaerated solution of 3.3 g (11.4 mmol) of 2-amino-5-nitro-6-(phenacylamino)-4-(3*H*)-pyrimidinone[3] in 200 mL of methanol, 50 mL of water, and 25 mL of glacial acetic acid was heated to 70 °C. Iron powder, 1.5 g (26.9 mmol), was added and the mixture vigorously stirred under argon for 90 min, at which time chromatography on the above Partisil SCX HPLC system showed complete consumption of the nitropyrimidine. Excess iron was removed with a magnet, the reaction kept at −15 °C overnight, and product obtained by filtration followed by a wash with cold water. Drying to constant weight under vacuum over $P_2O_5$ gave 2.57 g of 6-phenyl-7,8-dihydropterin which was chromatographically pure (monitored at 254 nm) except for a small residual of iron. UV spectra of pH 1, 7, and 14 of a chromatographically purified sample gave identical spectra with those previously reported (Pfleiderer & Mengel, 1971).

[3] 2-Amino-5-nitro-6-(phenacylamino)-4(3*H*)-pyrimidinone was prepared from 2-amino-6-chloro-5-nitro-4(3*H*)-pyrimidinone (Bailey & Ayling, 1983) and phenacylamine hydrochloride by the method of Pfleiderer and Mengel (1971).

The mass spectrum (FAB, positive ion) showed only $m/z$ 242 [$(M + H)^+$] above solvent background.

(*2*) *(R,S)-6-Phenyl-5,6,7,8-tetrahydropterin.* A solution of 1.81 g (7.5 mmol) of 6-phenyl-7,8-dihydropterin in 41 mL of trifluoroacetic acid (99+%) with 0.7 g of palladium (10%) on charcoal was stirred while being bubbled continuously with hydrogen at atmospheric pressure. The reaction was monitored by HPLC on Spherisorb $C_6$, 5 μm (25 × 0.4 cm), eluted at 1.5 mL/min with ammonium formate (0.05 M ammonia) containing 1 mM $Na_2EDTA$ (pH 3.5)/methanol (3:2) with detection by UV absorbance at 265 nm and electrochemical oxidation at 0.8 V vs Ag/AgCl. Hydrogenation was stopped at 130 min with the complete disappearance of starting material. At this point product contained 0.3% 6-cyclohexyl-5,6,7,8-tetrahydropterin. The reaction was filtered under argon through a Whatman 934 AH glass filter pad and washed with 20 mL of deaerated methanol. Product was precipitated from the filtrate with 5 mL of 4 M methanolic HCl and 200 mL of diethyl ether, collected by centrifugation, and dried under vacuum to give 1.95 g. Material for enzymatic reactions was purified by chromatography on Bakerbond 40-μm ODS preparative column (2.1 × 45 cm) eluted at 10.0 mL/min with methanol/1 M acetic acid containing 25 mM mercaptoethanol (15:85). The fraction containing the 6-phenyltetrahydropterin was passed through a short column of Chelex 100 under argon and rotary evaporated to an oil, 4 M methanolic HCl was added, and product was precipitated with ethyl ether. The mass spectrum (FAB, positive ion) showed $m/z$ 244 [$(M + H)^+$] and $m/z$ 141 at 100 and 20% abundance, respectively, after background subtraction. The molar extinction coefficient at 265 nm in 0.1 N HCl was determined to be 14 300 $M^{-1}$ $cm^{-1}$.

(*3*) *(R,S)-6-Cyclohexyl-5,6,7,8-tetrahydropterin.* (*R,S*)-6-Phenyl-5,6,7,8-tetrahydropterin (395 mg of unpurified material) was reduced in 12.0 mL of trifluoroacetic acid over 200 mg of $PtO_2$ (Adam's catalyst) with 45 psi $H_2$ with vigorous stirring for 42 h. At this time chromatography on the above Spherisorb $C_6$ system showed a 49:1 ratio of 6-cyclohexyl- to 6-phenyltetrahydropterin. Methanolic HCl (2 mL of 2 M) was added and the reaction quickly centrifuged at 15000*g* for 5 min. The supernate was similarly recentrifuged to remove traces of catalyst, and product was precipitated with 10 volumes of ethyl ether. Drying under vacuum over $P_2O_5$ gave 340 mg of light yellow powder. This material was purified as above on the Bakerbond 40-μm preparative column except, after elution of the 6-phenyltetrahydropterin, methanol was increased to 25% to speed recovery of the slower moving product. The desired fraction was rotary evaporated to an oil, redissolved in 2 mL of 2 M methanolic HCl, and precipitated with ethyl ether. The precipitate was recrystallized from 1 N HCl with acetonitrile to give colorless (*R,S*)-6-cyclohexyl-5,6,7,8-tetrahydropterin dihydrochloride. The mass spectrum (FAB, positive ion) showed $m/z$ 250 [$(M + H)^+$] as the base peak after background subtraction along with a few related ions at less than 10% relative abundance. The molar extinction coefficient at 265 nm in 0.1 N HCl was determined to be 14 600 $M^{-1}$ $cm^{-1}$.

*Tyrosine hydroxylase* was extracted from bovine brain striata. Brains were obtained fresh from slaughter and immediately dissected. The striata were homogenized with a Potter Elvejhem homogenizer in 3 volumes of 0.1 M potassium phosphate, pH 7.6. The homogenate was centrifuged at 100000*g* for 60 min. Comparison with a 10000*g* supernatant indicated that all of the tyrosine hydroxylase activity is recovered in the 100000*g* supernatant fraction. The yield of

activity was not increased by inclusion of 0.2% Triton X-100 in the homogenizing buffer or sonication for 2 min prior to centrifugation.

In order to measure tyrosine hydroxylase activity in unpurified preparations by HPLC analysis of the DOPA produced, it is necessary to prevent enzymatic decarboxylation. Although a number of inhibitors are available to inhibit aromatic amino acid decarboxylase, reductive deactivation of this pyridoxal phosphate dependent enzyme is an effective and convenient alternative. Treatment with sodium borohydride as described below blocks the conversion of DOPA to dopamine without affecting tyrosine hydroxylase activity, as assayed with (*R*,-*S*)-6-methyl-$PH_4$ or (*R*)-$BH_4$. A further advantage of this approach is that any inhibition of tyrosine hydroxylase by a decarboxylase inhibitor that may be emphasized by the use of a particular cofactor analogue is avoided. Although DOPA decarboxylase activity was decreased by over 99%, very high rates of hydroxylation were avoided in order to minimize dopamine formation toward the end of reactions. To inactivate DOPA decarboxylase, three equal aliquots of a 20 mM solution of $NaBH_4$ in 0.1 mM NaOH were added 1 min apart to the 100000*g* supernatant at 0 °C with gentle stirring to give a final concentration of 6 mM $NaBH_4$. The mixture was stirred for an additional 10 min. An equal volume of saturated ammonium sulfate in 0.1 M potassium phosphate, pH 7.6, was then added with agitation. After being stirred 10 min at 0 °C, the precipitated protein was sedimented by centrifugation at 15000*g* for 15 min at 2 °C. The supernatant was discarded and the precipitate taken up in 0.1 M potassium phosphate, pH 7.6, and dialyzed against three changes of the same buffer. The dialyzate was fast frozen and stored at −70 °C. This preparation, which was used as the source of enzyme, appears to be primarily unphosphorylated at the protein kinase A site (see below). Under the conditions used for determination of kinetic parameters, rates were linear with concentration of enzyme.

All kinetic parameters for tyrosine hydroxylase were measured in two different reaction conditions referred to as (i) "unphosphorylated" and (ii) "phosphorylated" tyrosine hydroxylase reactions.

(*i*) *Unphosphorylated Tyrosine Hydroxylase.* The $K_m$ for cofactor was determined by varying the concentration of analogue typically over the range of 0.02–5.0 mM, except for (*R*)-$BH_4$ which was extended down to 0.001 mM. Since all cofactors studied were prepared as their dihydrochloride salts, the resulting 0.04–10.0 mM strong acid significantly affects the pH of the medium. In order to maintain constant pH, HCl was added so that total HCl in all reactions was 10 mM, which was then compensated by use of a higher pH buffer. Standard reflections were run at a final pH of 7.15 (after all components were added) and 37 °C and contained per 100-μL final volume 20 nmol of tyrosine (except as noted), 2 μmol of sodium ascorbate, 25 units of superoxide dismutase, 250 units of catalase, 10 μmol of potassium phosphate, pH 7.4 (initial pH before addition of any other components), cofactor, and an amount of tyrosine hydroxylase which gave less than 0.07 nmol of DOPA per 100-μL reaction per minute as the fastest rate. All components but one (see Results) were preincubated together at 37 °C for 10 min before reactions were initiated. At 0.25, 10, 20, and 30 min after initiation aliquots were removed and added to an equal volume of 1 M trichloroacetic acid. The $K_m$ values for tyrosines as varied substrate were obtained similarly, but with cofactor fixed at 5 mM.

(*ii*) *Phosphorylated Tyrosine Hydroxylase.* It has been reported that crude preparations of tyrosine hydroxylase contain considerable phosphatase activity (Yamauchi & Fujisawa, 1979; Lazar et al., 1983; Nelson & Kaufman, 1987). In order to ensure complete cAMP-dependent phosphorylation, kinase activity was maintained throughout the course of a hydroxylase reaction. Further, as the amount of hydroxylase preparation (and therefore phosphatase activity) is increased in a reaction, increased protein kinase A activity is needed to promote complete cAMP-dependent phosphorylation (Figure 3). The required amount of protein kinase A was determined at a concentration of (*R*)-$BH_4$ (50 μM) which would saturate tyrosine hydroxylase phosphorylated with cAMP-dependent kinase, but which would produce insignificant rates with remaining unphosphorylated enzyme. The maximum observable rate could be obtained either by increasing protein kinase activity or by decreasing the concentration of hydroxylase (Figure 3). On the basis of the relationship established with (*R*)-$BH_4$ (which has been shown to cause activation of some brain phosphatases; Nelson & Kaufman, 1987), all of the data presented below were obtained with an amount of hydroxylase preparation and phosphorylating reagents found to promote 90% or more conversion to the high-affinity form. Each $K_m$ determination included a set of 6-methyl-$PH_4$ control reactions which served to monitor the degree of phosphorylation and enzyme activity over the span of the experiments.

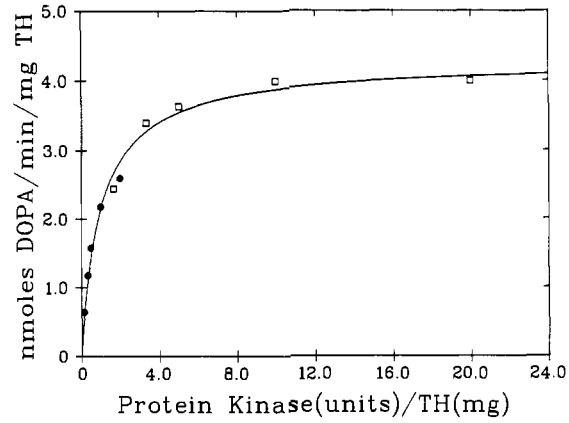


FIGURE 3: Effect of the ratio of protein kinase to tyrosine hydroxylase on the extent of cAMP-dependent phosphorylation. Protein kinase is in nmol units. Tyrosine hydroxylase (TH) is expressed as mg of protein of the minimally purified bovine striatal preparation. Reactions were at pH 7.15 and 37 °C and contained per 100-μL final volume 10 nmol of tyrosine, 5 nmol of (*R*)-$BH_4$, 2 μmol of sodium ascorbate, 25 units of superoxide dismutase, 250 units of catalase, 10 μmol of potassium phosphate, 0.4 μmol of $MgCl_2$, 0.12 μmol of ATP, 6 nmol of cAMP, 0.04–0.5 nmol units of protein kinase A, and 25 μg (□) or 250 μg (●) of tyrosine hydroxylase at each concentration of protein kinase. Aliquots were removed from each reaction as a function of time and analyzed for DOPA as described under Materials and Methods.

A double check for the effectiveness of the cAMP-dependent phosphorylation was the measurement of cofactor $K_m$ values over a broad range of cofactor concentration, followed by analysis of the data with nonlinear least squares fitted to a Michaelis–Menten equation modified for simultaneous activity of two independent enzyme forms (see below). The effect of adding inadequate protein kinase A is illustrated in Figure 4, curve b, which contained, in an otherwise standard phosphorylated tyrosine hydroxylase reaction, 0.012 nmol unit protein kinase A, instead of the standard 0.25 nmol unit in curve a. No phosphorylating reagents were included for curve c. Using the equation given below, tyrosine hydroxylase was calculated to be 30% in the phosphorylated form in curve b.

Standard phosphorylated tyrosine hydroxylase reactions were at a final pH of 7.15 (after all components were added) at 37 °C and contained per 100-μL final volume 20 nmol of

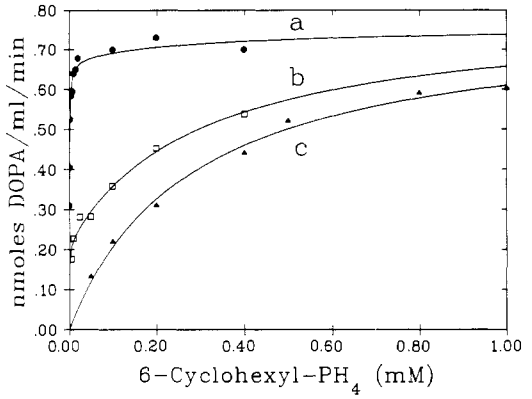


FIGURE 4: Michaelis–Menten curves for 6-cyclohexyl-$PH_4$ with tyrosine hydroxylase (a) >90% phosphorylated with protein kinase A, (b) 30% phosphorylated, and (c) unphosphorylated. (a) Standard phosphorylated tyrosine hydroxylase reaction conditions. (b) The same as (a) except with 20 times less protein kinase A. (c) Standard unphosphorylated reaction conditions. Data were fitted by nonlinear regression (c) to a simple Michaelis–Menten equation and (a and b) to the Michaelis–Menten equation modified for simultaneous activity of two different enzyme forms (see Materials and Methods).

Table I: Michaelis Constants for Tyrosine with Unphosphorylated or Phosphorylated Tyrosine Hydroxylase[a]

| | unphosphorylated | | | phosphorylated | | |
|---|---|---|---|---|---|---|
| cofactor | [cof] (mM) | $K_m$ ($\mu$M) | rel $V_{max}$[b] | [cof] (mM) | $K_m$ ($\mu$M) | rel $V_{max}$[b] |
| 6-monosubstituted | | | | | | |
| (*R*)-$BH_4$ | | | | 0.05 | 7–90[c] | >0.48 |
| 6-hydroxymethyl-$PH_4$ | | | | 0.2 | 20–90[c] | >0.4 |
| 6-methyl-$PH_4$ | 5.0 | 60 | 0.18 | 0.4 | 45 | 1.0 |
| 6-ethyl-$PH_4$ | 5.0 | 22 | 0.3 | 0.02 | 7 | 0.32 |
| 6-phenyl-$PH_4$ | | | | 0.02 | 33 | 0.22 |
| 6,6-disubstituted | | | | | | |
| 6,6-dimethyl-$PH_4$ | 5.0 | 30 | 0.034 | 0.4 | 29 | 0.26 |
| 6-ethyl-6-methyl-$PH_4$ | 5.0 | 36 | 0.086 | 0.1 | 30 | 0.26 |
| 6-phenyl-6-methyl-$PH_4$ | | | | 0.1 | 120 | 0.22 |

[a] Tyrosine $K_m$ values were measured at atmospheric $O_2$, pH 7.15, 37 °C, and cofactor at the concentrations indicated in the table. [b] The rate with 6-methyl-$PH_4$ and phosphorylated enzyme is defined as 1. [c] Substrate inhibited by tyrosine; rel $V_{max}$ is calculated from data at concentrations of tyrosine below the observed maximum.

tyrosine (except as noted), 2 $\mu$mol of sodium ascorbate, 25 units of superoxide dismutase, 250 units of catalase, 10 $\mu$mol of potassium phosphate, pH 7.18 (initial pH before addition of any other components), 0.25 nmol unit of 3′,5′-cAMP-dependent protein kinase, 0.4 $\mu$mol of $MgCl_2$, 0.3 nmol of 3′,5′-cAMP, 25 $\mu$g of tyrosine hydroxylase, and 0.12 $\mu$mol 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 initiated by addition of cofactor. Aliquots were removed at 0.25, 5, 10, and 20 min and added to an equal volume of 1 M trichloroacetic acid. Because of the lower range of cofactor concentrations, special precautions to ensure a fixed pH by addition of constant acid were not required. The $K_m$ values for tyrosine as varied substrate were performed with cofactor at the concentrations specified in Table I. To ensure that only the cAMP-dependent site was phosphorylated (serine-40; Campbell et al., 1986), control reactions were run which contained $MgCl_2$ and ATP but no added cAMP or protein kinase. Under these conditions, the $K_m$ curve for (*R*)-$BH_4$ was identical with that for unphosphorylated tyrosine hydroxylase, indicating that the phosphorylation is cAMP specific.

*Quantitation of DOPA.* Precipitated protein was removed from reaction samples by centrifugation, and the supernatant was assayed for DOPA by HPLC with fluorescence detection, by adaptation of a published procedure (Bailey & Ayling, 1980). A Spherisorb (3 $\mu$m) ODS column (15 × 0.46 cm) was utilized with ammonium formate (30 mM in ammonia), pH 3.3, containing 1 mM $Na_2EDTA$ as the mobile phase. Fluorescence detection was with excitation at 282 nm and emission at 325 nm. The lower limit of detection is about 1 pmol (signal/noise = 2). Rates were determined from the linear portion of the progress curves (see Results). HPLC analyses were performed with a Spectra-Physics 8700 delivery system. A Gilson 231/401 autoinjector equipped with a water-cooled sample rack was used for automated analyses of DOPA. Fluorescence was monitored with the detector previously described (Bailey & Ayling, 1980). (Comparable results can be obtained with the Perkin-Elmer LS-4.)

*Data Analysis.* Michaelis constants and maximum velocities were calculated with a nonlinear regression program (MINSQ, Micromath, Salt Lake City, UT). Fitting data to the hyperbolic Michaelis–Menten equation rather than to a Lineweaver–Burk linear equation was particularly valuable (i) for the high $K_m$ values with unphosphorylated tyrosine hydroxylase in which it was necessary to make most of the measurements at concentrations below $K_m$ and (ii) for multiple forms of the enzyme. In the latter case, data were fitted to

$$V = \frac{SV_{max\,1}}{S + K_{m\,1}} + \frac{SV_{max\,2}}{S + K_{m\,2}}$$

in which $K_{m\,1}$ and $K_{m\,2}$ are the Michaelis constants and $V_{max\,1}$ and $V_{max\,2}$ are the maximum velocity contributions of the phosphorylated and unphosphorylated components of the reaction. $K_{m\,2}$ was fixed at the value determined independently from the unphosphorylated reactions. The percent phosphorylation can be obtained after optimization of the remaining three free parameters as $100(1 - V_{max\,2}/V_{max}^{u})$, where $V_{max}^{u}$ is the maximum velocity in the fully unphosphorylated reaction. The $V_{max}$ of totally phosphorylated enzyme is then $V_{max\,1}V_{max}^{u}/(V_{max}^{u} - V_{max\,2})$.

## Results

*Reactions vs Time.* The progress curves of reactions employing unphosphorylated tyrosine hydroxylase were observed to accelerate over the first 10 min. Afterward linear rates were maintained under most circumstances up to at least 60 min. This lag, which has been reported for rat striatal tyrosine hydroxylase assayed with (*R*)-$BH_4$ at neutral pH (Miller & Lovenberg, 1985), was observed with all cofactors investigated. It was significantly diminished if enzyme and cofactor were both included in the 10-min preincubation at 37 °C, and reaction initiated with tyrosine rather than with cofactor. The extent of lag was found to be further decreased at cofactor concentrations greater than $K_m$. However, at the higher concentrations the rates with most cofactors began to drop after about 20–30 min. The combination of the lag and the decline in rate at high cofactor places a limitation on the accuracy of the measurement of high $K_m$ values. Rates were obtained by measurement of the fastest part of the progress curve which occurred between 10 and 30 min.

With (*R,S*)-6-phenyltetrahydropterin as cofactor, the electron-withdrawing phenyl group promotes a high kinetic acidity of the hydrogen at the 6-position. Thus, despite the use of reducing agents, quinoid (*R,S*)-6-phenyldihydropterin rapidly tautomerizes to 6-phenyl-7,8-dihydropterin.[2] Not only is reduced cofactor lost, but also a potential hydroxylase inhibitor accumulates (Nagatsu et al., 1972; Ayling & Bailey, 1983). With this cofactor, rates began to decrease after 20 min at all concentrations.

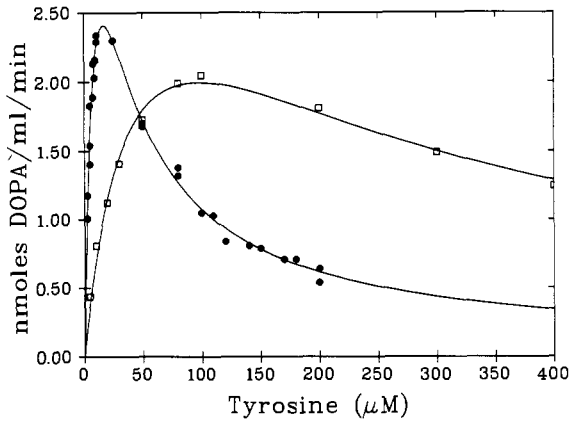


FIGURE 5: Substrate inhibition of phosphorylated tyrosine hydroxylase by tyrosine, with (*R*)-$BH_4$, 0.05 mM (●), or 6-hydroxymethyl-$PH_4$, 0.2 mM (□), as cofactor.

No lag was seen with any cofactor, regardless of the order of addition of reaction components, when tyrosine hydroxylase was phosphorylated with cAMP-dependent protein kinase. Initial rates were therefore used for determination of kinetic parameters. Reactions proceeded linearly for approximately 10–20 min followed by a slow decline at all concentrations of each cofactor.

*$K_m$ for Tyrosine.* Unphosphorylated tyrosine hydroxylase was analyzed in the presence of much higher concentrations of cofactor than phosphorylated enzyme so that, although apparent values are still obtained, these may more closely reflect true $K_m$ values.

The $K_m$ for tyrosine with (*R,S*)-6-methyl-$PH_4$ as cofactor of 60 μM (Table I), even though assayed with 5 mM cofactor, is within the range of literature values reported for unphosphorylated tyrosine hydroxylase at pH 6 (Lloyd & Kaufman, 1975; Vulliet et al., 1980; Lazar et al., 1982). Upon cAMP-dependent phosphorylation, the value drops only slightly, again consistent with these reports that phosphorylation of the hydroxylase has little impact on $K_m$ for substrate with the 6-monomethyl cofactor.

Substrate inhibition by tyrosine was observed with phosphorylated hydroxylase not only with (*R*)-$BH_4$ but also with (*R,S*)-6-hydroxymethyl-$PH_4$ (Figure 5). With (*R*)-$BH_4$ the highest rate observed occurred between 10 and 20 μM tyrosine. Reaction velocities and the profile of the Michaelis–Menten curve were similar with (*R,S*)-$BH_4$. In either case, by using only the data collected at low tyrosine, a $K_m$ of 7 μM can be estimated, a value close to those reported with (*R,S*)-$BH_4$ (Kaufman, 1975; Lazar et al., 1982). With 6-hydroxymethyl-$PH_4$ the highest rate was at 100 μM tyrosine. By ignoring tyrosine concentrations above 80 μM, a $K_m$ of 20 μM can be obtained. However, such analyses may not be justified in the presence of such severe substrate inhibition. The entire $V$ vs $S$ curves were fitted to the equations describing a number of mechanisms of substrate inhibition (Segal, 1975). Several models were found to closely fit the data which suggested that the apparent $K_m$ might be considerably higher. However without knowledge of the appropriate mechanism of substrate inhibition, the closeness of any particular fit alone is not sufficient to provide a reliable $K_m$ value. The range of possible values is listed in Table I.

None of the other cofactor analogues studied gave tyrosine substrate inhibition. With two exceptions, these gave $K_m$ values that were close to those found with (*R,S*)-6-methyl-$PH_4$ and did not change upon cAMP-dependent phosphorylation (Table I). In contrast to the typical result, $K_m$ for tyrosine with (*R,S*)-6-ethyl-$PH_4$ dropped to the low value of 7 μM with phosphorylated hydroxylase, a 3-fold change from that of unphosphorylated enzyme. The second exception is the 120 μM $K_m$ for tyrosine with (*R,S*)-6-phenyl-6-methyl-$PH_4$.

*Cofactor Kinetic Parameters with Unphosphorylated Tyrosine Hydroxylase.* Data for the Michaelis–Menten curves were collected at a tyrosine concentration of 0.2 mM for all cofactors except (*R*)-$BH_4$, with which 0.1 mM tyrosine was used. The former concentration is sufficient to produce greater than 80% saturation in tyrosine for most cofactors, except (*R,S*)-6-phenyl-6-methyl-$PH_4$ (see Table I) with which this would have been only approximately twice $K_m$. These levels of tyrosine are also close to those of brain tissue estimated to range between 0.11 and 0.15 mM (Kaufman & Kaufman, 1985).

In several cases with unphosphorylated tyrosine hydroxylase the measured $K_m$ was so high that an accurate value was difficult to determine (see Reaction vs Time). Even with these cofactors [i.e., (*R*)-$BH_4$, (*R,S*)-6-hydroxymethyl-$PH_4$, (*R,S*)-6-methoxymethyl-$PH_4$, and (*R,S*)-6-benzyl-6-methyl-$PH_4$] a good value of $V_{max}/K_m$ can be quantitated from the slope of the lower points in the curve. For (*R*)-$BH_4$ the value of 5 mM is consistent with extrapolations from measurements at other pHs and tyrosine concentrations where the $K_m$ is lower and therefore can be more accurately determined.[4] This is in fairly close agreement with the value reported by Miller and Lovenberg (1985) for (*R*)-$BH_4$ with unphosphorylated rat striatal tyrosine hydroxylase in neutral phosphate buffer.

The rapid but limited rise in hydroxylation rate at very low (*R*)-$BH_4$ concentrations that has been ascribed to a small percentage of a high-activity form of tyrosine hydroxylase in unphosphorylated preparations (Miller & Lovenberg, 1985) was observed in the current study. The results were analyzed by nonlinear regression with both single- and double-$K_m$ Michaelis–Menten equations. Despite the occurrence of the two distinct $K_m$ values, the contribution of the high-affinity form was so small (only 1–2%) that the $K_m$ and $V_{max}$ of the major low-affinity form could be obtained as accurately by use of the equation for a single $K_m$ as from the double-$K_m$ equation (see Materials and Methods). Thus, all of the data for unphosphorylated tyrosine hydroxylase with the cofactor analogues were similarly fitted to the single-$K_m$ Michaelis–Menten equation.

With unphosphorylated tyrosine hydroxylase the ranking of cofactor affinity, with one exception, directly correlated to the hydrophobicity of the 6-substituent, regardless of mono- or disubstitution (Table II). The $K_m$ values ranged from 5 mM or greater with (*R*)-$BH_4$ and (*R,S*)-6-hydroxymethyl-$PH_4$ to 0.3 mM for (*R,S*)-6-cyclohexyl-$PH_4$ and 6-phenyl-$PH_4$. The exception was (*R,S*)-6-benzyl-6-methyl-$PH_4$ which was found to have one of the highest $K_m$ values, approximately 4 mM. This is a 10-fold increase over that of (*R,S*)-6-phenyl-6-methyl-$PH_4$ despite an additional methylene group. By comparison of (*R,S*)-6-methyl-, (*R,S*)-6-ethyl-, and (*R,S*)-6-phenyl-$PH_4$ with their respective disubstituted analogues, the extra 6-methyl group either slightly decreased the $K_m$ for the first two compounds or slightly increased it for the third.

The maximum velocities for all of the cofactor analogues with unphosphorylated tyrosine hydroxylase varied over an 8-fold range from a high with the natural cofactor, (*R*)-$BH_4$ (despite considerable substrate inhibition), to a low with 6,6-dimethyl-$PH_4$. On average, the 6-monosubstituted analogues were 3.5 times faster than the 6,6-disubstituted analogues. The slowest of the monosubstituted cofactors was

[4] J. E. Ayling, et al., unpublished results.

Table II: Kinetic Parameters for (*R*)-$BH_4$ and Analogues with Unphosphorylated or Phosphorylated Tyrosine Hydroxylase[a]

| cofactor | unphosphorylated | | | phosphorylated | | | $V_{max}$(P)/$V_{max}$(U)[d] | $K_m$(U)/$K_m$(P)[d] |
|---|---|---|---|---|---|---|---|---|
| | $K_m$ (mM) | rel $V_{max}$[b] | rel $V_{max}/K_m$ | $K_m$ (mM) | rel $V_{max}$[b] | rel $V_{max}/K_m$ | | |
| 6-monosubstituted | | | | | | | | |
| (*R*)-$BH_4$ | ~5 | ~0.5[c] | 0.1 | 0.003 | 0.24[c] | 80 | | 1700 |
| 6-hydroxymethyl-$PH_4$ | >5 | | 0.03 | 0.02 | 0.4[c] | 20 | | >250 |
| 6-methyl-$PH_4$ | 3 | 0.28 | 0.09 | 0.06 | 1 | 17 | 3.5 | 50 |
| 6-methoxymethyl-$PH_4$ | >2 | | ~0.02 | 0.003 | 0.39 | 130 | | >670 |
| 6-ethyl-$PH_4$ | 2 | 0.39 | 0.19 | 0.001 | 0.31 | 310 | 0.8 | 2000 |
| 6-phenyl-$PH_4$ | 0.3 | ~0.4 | ~1.2 | 0.0008 | 0.24 | 300 | ~0.6 | 380 |
| 6-cyclohexyl-$PH_4$ | 0.3 | 0.18 | 0.6 | 0.0006 | 0.18 | 300 | 1 | 500 |
| 6,6-disubstituted | | | | | | | | |
| 6,6-dimethyl-$PH_4$ | 1.4 | 0.06 | 0.04 | 0.04 | 0.24 | 6 | 4 | 35 |
| 6-ethyl-6-methyl-$PH_4$ | 1.4 | 0.11 | 0.08 | 0.007 | 0.22 | 32 | 2 | 200 |
| 6-propyl-6-methyl-$PH_4$ | 0.5 | 0.11 | 0.22 | 0.004 | 0.2 | 50 | 1.8 | 130 |
| 6-phenyl-6-methyl-$PH_4$ | 0.5 | 0.1 | 0.2 | 0.008 | 0.13 | 16 | 1.3 | 50 |
| 6-benzyl-6-methyl-$PH_4$ | ~4 | ~0.13 | 0.03 | 0.014 | 0.10 | 7 | ~0.8 | ~290 |

[a] Cofactor $K_m$ values were measured at atmospheric oxygen, pH 7.15, 37 °C, and 0.2 mM tyrosine, except for (*R*)-$BH_4$ in which case 0.1 mM tyrosine was used. [b] The rate with 6-methyl-$PH_4$ and phosphorylated enzyme is defined as 1. [c] Substrate inhibited by tyrosine. [d] (P) = phosphorylated with protein kinase A; (U) = unphosphorylated.

found to have a $V_{max}$ 50% higher than that of the fastest 6,6-disubstituted compound. Within each group of mono- or disubstituted compounds, structural changes resulted in only approximately 2–3-fold variations in maximum velocity.

The affinity of a cofactor analogue with unphosphorylated tyrosine hydroxylase was not well correlated with its $V_{max}$, as can be seen by the greater spread of $V_{max}/K_m$ than of either parameter independently (Table II). This ratio varied over a 40-fold range with (*R,S*)-6-phenyl-$PH_4$ and (*R,S*)-6-cyclohexyl-$PH_4$ having the highest values (about 5 and 2.5 times above average, respectively) and (*R,S*)-6-hydroxymethyl-$PH_4$ and (*R,S*)-6-benzyl-6-methyl-$PH_4$ having the lowest values (both about 8 times below average). Interestingly, both (*R*)-$BH_4$ and (*R,S*)-6-methyl-$PH_4$ were considerably below average in this respect, their higher $V_{max}$ values failing to compensate for their high $K_m$ values.

*Cofactor Kinetic Parameters with cAMP-Dependent Phosphorylated Tyrosine Hydroxylase.* The most profound effect of phosphorylation of tyrosine hydroxylase by protein kinase A is a large decrease in $K_m$ for cofactor (Table II). Although a decrease in cofactor $K_m$ upon cAMP-dependent phosphorylation has been reported previously for 6-methyl-$PH_4$ and $BH_4$, the current study demonstrates increases in affinity between 35- and 2000-fold, dependent on the nature of the 6-position substituents. As with unphosphorylated enzyme, 6-substituent hydrophobicity is a major factor in determining $K_m$, but two significant deviations from this pattern are seen. On progressive extension of nonpolar bulk in (*R,S*)-6-methyl-, (*R,S*)-6-ethyl-, (*R,S*)-6-phenyl-, and (*R,S*)-6-cyclohexyl-$PH_4$ a continuous decrease in $K_m$ is found from 60 to 0.6 μM, with most of the drop occurring with the addition of the second carbon unit. With phosphorylated hydroxylase, however, 6,6-disubstituted analogues do not fit smoothly into this sequence as they do with unphosphorylated enzyme. With 6,6-dimethyl-$PH_4$, the simplest 6,6-disubstituted compound, the extra methyl causes a slight increase in affinity over that of (*R,S*)-6-methyl-$PH_4$. Further decreases in $K_m$ below that of 6,6-dimethyl-$PH_4$ are found as the second 6-substituent is extended from ethyl to propyl, although the trend is then reversed with even larger groups. A comparison of (*R,S*)-6-ethyl- and (*R,S*)-6-phenyl-$PH_4$ with their 6,6-disubstituted counterparts shows that the latter have 7–10-fold higher $K_m$ values.

The second exception to the trend of increasing $K_m$ with decreasing hydrophobicity was found with (*R*)-$BH_4$ and (*R,S*)-6-hydroxymethyl-$PH_4$. The natural cofactor was found to have a $K_m$ value of 3 μM[5] (cf. 8 μM for phosphorylated rat striatal tyrosine hydroxylase; Miller & Lovenberg, 1985), which although still 5-fold higher than that of (*R,S*)-6-cyclohexyl-$PH_4$ is 20-fold lower than that of (*R,S*)-6-methyl-$PH_4$. Thus, although having nearly the lowest affinity for unphosphorylated tyrosine hydroxylase, (*R*)-$BH_4$ is among the cofactors with highest affinity after cAMP-dependent phosphorylation. The ratio of $K_m$(unphosphorylated)/$K_m$(phosphorylated) of 1700 is exceeded only by that of (*R,S*)-6-ethyl-$PH_4$. An analogue with a structure intermediate between (*R*)-$BH_4$ and (*R,S*)-6-methyl-$PH_4$, (*R,S*)-6-hydroxymethyl-$PH_4$, was found to have an intermediate $K_m$ of 20 μM.

The range of maximum velocities with cAMP-dependent phosphorylated tyrosine hydroxylase was similar to that with unphosphorylated enzyme. Despite the 10-fold variation, 8 out of the 12 cofactors investigated had relative $V_{max}$'s between 0.2 and 0.4. The effect of phosphorylation on $V_{max}$ was not uniform; increases of up to 4-fold [(*R,S*)-6-methyl- and 6,6-dimethyl-$PH_4$] and decreases of 0.6 [(*R,S*)-6-phenyl-$PH_4$] were measured. However, although (*R,S*)-6-methyl-$PH_4$ has the highest observed $V_{max}$, both (*R*)-$BH_4$ and (*R,S*)-6-hydroxymethyl-$PH_4$ may have equal or higher theoretical $V_{max}$'s when substrate inhibition by tyrosine and $O_2$ are taken into account.

The $V_{max}/K_m$ for all cofactors is greatly increased upon cAMP-dependent phosphorylation of tyrosine hydroxylase, due mainly to the lowering of $K_m$. Even the lowest values [(*R,S*)-6-benzyl-6-methyl-$PH_4$ and 6,6-dimethyl-$PH_4$] are five times above the highest value found with unphosphorylated enzyme. In this view of cofactor effectiveness, (*R,S*)-6-methyl-$PH_4$ despite its high maximum velocity ranks with the poorest cofactors for phosphorylated hydroxylase due to its high $K_m$. Two 6,6-disubstituted compounds, (*R,S*)-6-ethyl-6-methyl- and (*R,S*)-6-propyl-6-methyl-$PH_4$, have $V_{max}/K_m$ values that are twice that of (*R,S*)-6-methyl-$PH_4$. Even with the handicap of substrate inhibition, the natural cofactor, (*R*)-$BH_4$, has a value twice again as high as these latter analogues but still considerably below the top ranking (*R,S*)-6-ethyl-, (*R,S*)-6-phenyl-, and (*R,S*)-6-cyclohexyl-$PH_4$.

## Discussion

Most cofactor structure–function relationships with tyrosine hydroxylase have been carried out at pH 6. However, a substantial shift in enzyme properties with increasing pH has

[5] The values for $K_m$ and $V_{max}$ with RS-$BH_4$ were similar.

been reported by several laboratories (Markey et al., 1980; Pollock et al., 1981; Pradhan et al., 1981; Lazar et al., 1982; Miller et al., 1985). Thus, the current studies were conducted at the pH of striatum, pH 7.15 (Hope et al., 1987). Further, even when similar pH, tissue source, and tyrosine concentration are being compared, literature values for $K_m$ and relative $V_{max}$ for $BH_4$ and 6-methyl-$PH_4$ frequently vary. Several explanations for many of the inconsistencies are possible, among which may be the state of purification of the enzyme. For example, Bullard and Capson (1983) reported a significant shift in $K_m$ upon purification of bovine striatal tyrosine hydroxylase. The purified material not only showed none of the biphasic kinetic behavior observed by other workers but also gave an approximately 3-fold lower $K_m$ for (*R*,*S*)-$BH_4$ compared to the crude supernatant fraction. Oka et al. (1982) have isolated at least two kinetically distinct forms of bovine tyrosine hydroxylase, and phosphorylated and dephosphorylated rat striatal enzyme has been separated on DEAE-cellulose (Sze et al., 1983). Recently, work on murine neuronal cells in culture has shown that in the basal state this system consists of a variety of phosphorylated forms of the hydroxylase (Kapatos, 1987). The situation is potentially even more complicated by the presence of isozymes. Multiple primary sequences have already been detected for human tyrosine hydroxylase (Grima et al., 1987; Kaneda et al., 1987; O'Malley et al., 1987). Since a complete characterization of ratios of tyrosine hydroxylase subtypes in bovine striatum is currently unavailable, only a minimally purified preparation (0–50% ammonium sulfate precipitate) was used. Although this enzyme may still contain phosphate at some positions, the high $K_m$ values observed with all analogues suggest that it is not significantly phosphorylated at the serine residue acted on by the cAMP-dependent protein kinase.

Substantial decreases in $K_m$ for cofactor upon phosphorylation of tyrosine hydroxylase with protein kinase A were observed with all analogues studied. Changes in affinity upon phosphorylation have been reported previously for $BH_4$ and 6-methyl-$PH_4$ (Lloyd & Kaufman, 1975; Lovenberg et al., 1975; Yamauchi & Fujisawa, 1979; Markey et al., 1980; Vulliet et al., 1980; Pollock et al., 1981; Lazar et al., 1982; Miller & Lovenberg, 1985). However, the data in Table II with a more extensive set of cofactors show that the effect of phosphorylation depends upon the nature of the 6-substituent(s), with $K_m$ shifts ranging from 35- to 2000-fold. Several striking patterns are evident.

The ranking of cofactor $K_m$ values indicates that different factors are emphasized in cofactor binding to unphosphorylated tyrosine hydroxylase in comparison to enzyme phosphorylated with cAMP-dependent kinase. With unphosphorylated enzyme, with the exception of (*R*,*S*)-6-benzyl-6-methyl-$PH_4$, there is a direct correlation with side-chain hydrophobicity. As either the hydroxyl groups of (*R*)-$BH_4$ are removed or extra methylene units are added, the $K_m$ drops. After phosphorylation with cAMP-dependent protein kinase, (*R*,*S*)-6-hydroxymethyl-$PH_4$ and the natural cofactor, (*R*)-$BH_4$, acquire a higher affinity than (*R*,*S*)-6-methyl-$PH_4$. In contrast to unphosphorylated tyrosine hydroxylase, there appear to be two modes of decreasing the $K_m$: either by the addition of hydroxyl groups or by increasing the number of carbon atoms in one of the 6-substituents. Overall, a 100-fold drop is observed between the $K_m$ for (*R*,*S*)-6-methyl-$PH_4$ and that for (*R*,*S*)-6-cyclohexyl-$PH_4$, an order of magnitude larger change than with unphosphorylated enzyme. Almost all of this decrease is obtained by extension of the alkyl group by only one methylene unit to (*R*,*S*)-6-ethyl-$PH_4$. The lower $K_m$ for (*R*,*S*)-6-ethyl-$PH_4$ compared with 6-methoxymethyl-$PH_4$ suggests that it is the lipophilic character of the side chain and not just increased size that is recognized. Unphosphorylated hydroxylase appears to require slightly larger substituents to achieve its minimum $K_m$ for cofactor.

The 6,6-disubstituted analogues have a number of characteristics which distinguish them from their monosubstituted counterparts, such as their unusual pattern of affinity for tyrosine hydroxylase phosphorylated with protein kinase A. For example, although 6,6-dimethyl-, (*R*,*S*)-6-ethyl-6-methyl-, and (*R*,*S*)-6-propyl-6-methyl-$PH_4$ constitute a series of homologues with decreasing $K_m$ with increasing hydrophobicity, the second compound has a value that is 7 times higher than that of (*R*,*S*)-6-ethyl-$PH_4$. Several factors may be involved in the effect of disubstitution. Since the 6,6-disubstituted compounds have both the axial and equatorial positions simultaneously occupied, the higher than expected $K_m$ for the three simplest disubstituted cofactors may indicate small interference by even a methyl group located in the nonpreferred orientation. The kinetics of the 6,6-disubstituted cofactors may also be related to an additional energy requirement for placing the side chain into an unfavorable position. Conformational analysis using MM2 plus PI (Burkert & Allinger, 1982) indicates that a barrier to rotation of some 6-substituents can be introduced by the presence of the second 6-methyl group.[2]

The differentiation of mono- from disubstituted cofactors is another indication, in addition to recognition of side-chain hydroxyls, of significant alterations in cofactor specificity caused by phosphorylation. With unphosphorylated enzyme both classes of cofactors form a continuous series of $K_m$ values based solely on total side-chain hydrophobicity. The poor affinity of (*R*,*S*)-6-benzyl-6-methyl-$PH_4$ for either form of tyrosine hydroxylase may not be a result of only its disubstitution pattern. In comparison to phenyl, the extra methylene unit of the benzyl group puts substantially more bulk out of the plane of the pteridine ring for most conformations.

The velocity data in Table II show that (at near saturating concentrations of tyrosine) the effect of cAMP-dependent phosphorylation is quite variable, increasing $V_{max}$ by up to 4-fold with some analogues and decreasing $V_{max}$ by 40% with others. The maximum velocities of (*R*)-$BH_4$ and (*R*,*S*)-6-hydroxymethyl-$PH_4$ cannot be precisely compared with the other cofactors, since both are strongly substrate inhibited by tyrosine (see below). In addition, it has been shown that the natural cofactor is inhibited by high (e.g., atmospheric) oxygen concentrations (Fisher & Kaufman 1972). Excluding (*R*)-$BH_4$, and (*R*,*S*)-6-hydroxymethyl-$PH_4$, there is a slight negative correlation of $V_{max}$ with hydrophobic bulk with phosphorylated tyrosine hydroxylase.

Within the 6-monosubstituted tetrahydropterins, $V_{max}$ varies only over a 2–3-fold range with unphosphorylated tyrosine hydroxylase. The entire class of disubstituted analogues also has maximum velocities in a narrow range, but on average they are 3–4 times slower than their monosubstituted counterparts. A possible explanation for these low rates of DOPA production is that with unphosphorylated tyrosine hydroxylase the disubstitution pattern may cause an uncoupled reaction. That is, more cofactor may be consumed than DOPA produced. Thus far, uncoupled reactions of tyrosine hydroxylase have only been reported for 7-substituted tetrahydropterins, e.g., 7-methyl- and 6,7-dimethyl-$PH_4$ (Shiman et al., 1971). With phenylalanine hydroxylase, only (*R*,*S*)-6-benzyl-6-methyl-$PH_4$ and (*R*,*S*)-6-phenyl-6-methyl-$PH_4$ have been shown to be uncoupled cofactors among this same set of 6,6-disubstituted compounds.[2] After phosphorylation with protein kinase A,

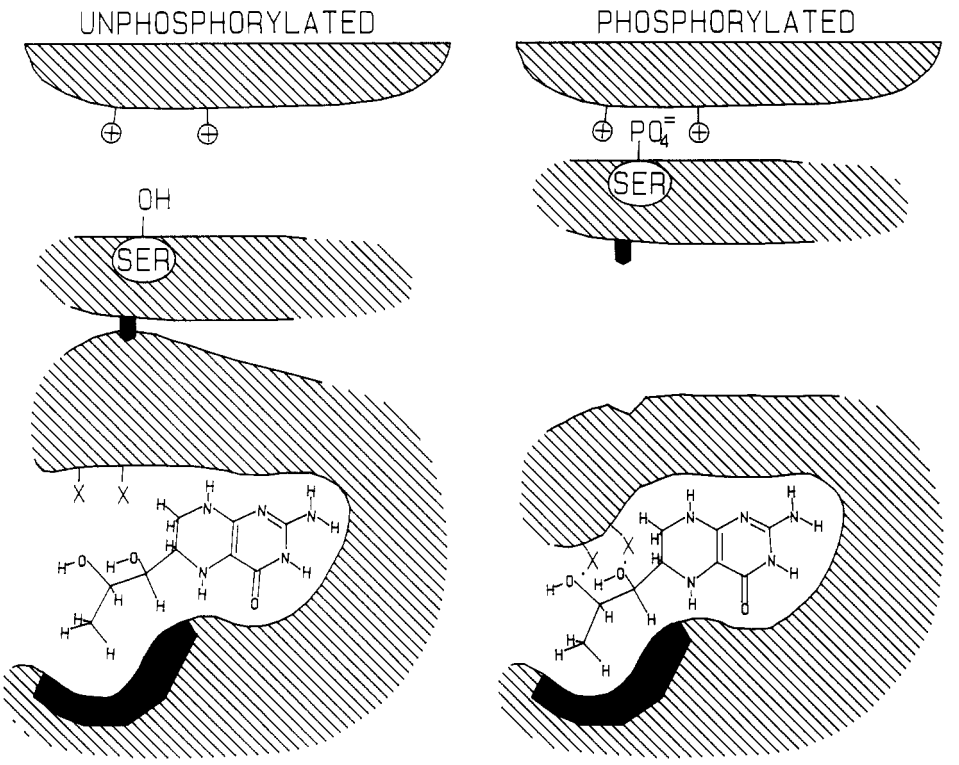


FIGURE 6: Proposed model of the influence of cAMP-dependent phosphorylation on the cofactor binding domain of tyrosine hydroxylase. Phosphorylation allows a relaxation of the region surrounding the tetrahydropterin side chain promoting simultaneous interactions between protein residues recognizing the hydroxyl groups (X) and the three carbons of the propyl chain (black area). These interactions in the unphosphorylated state are decreased but are still observable in the form of substrate inhibition induced by $BH_4$ and the influence, though to a lesser degree, of hydrophobic 6-substituents. Several mechanisms for transmitting the influence of phosphorylation to the catalytic site are possible. In the one shown, a salt link is made between positive residues on another part of the polypeptide chain and the phosphoserine on the N-terminal region pulling it away from the catalytic site, a change that can also be obtained to some extent by proteolytic removal of the N-terminal peptide.

the disparity between monosubstituted and at least the shorter chain disubstituted cofactors largely disappears, suggesting that phosphorylated tyrosine hydroxylase may be able to promote more tightly coupled reactions with a broader variety of structures.

The lower $K_m$ for cofactor that is observed with many of the purely hydrocarbon substituents than with (*R*)-$BH_4$, even with hydroxylase phosphorylated by cAMP-dependent protein kinase, suggests that some of the energy of binding of the natural cofactor may be used to facilitate events other than just a tight association. The presence of at least one side-chain hydroxyl group seems to be critical for enabling inhibition by high tyrosine concentrations. None of the purely hydrocarbon 6-mono- or 6,6-disubstituted cofactors produced substrate inhibition by tyrosine with either unphosphorylated or cAMP-dependent phosphorylated tyrosine hydroxylase. Inhibition by tyrosine with $BH_4$ as cofactor has been reported for unphosphorylated bovine adrenal enzyme at pH 6 (Shiman et al., 1971) and for both unphosphorylated and protein kinase A phosphorylated bovine striatal enzyme at pH 7 (Lazar et al., 1982). Using unphosphorylated bovine adrenal enzyme assayed at pH 6, Kato et al. (1980) found that this phenomenon was restricted to $BH_4$ and that (*R*,*S*)-6-hydroxymethyl-$PH_4$ and other polyhydroxyalkyl isomers gave a simple hyperbolic curve. In the current study with cAMP-dependent phosphorylated striatal enzyme assayed at physiological pH, substrate inhibition with (*R*,*S*)-6-hydroxymethyl-$PH_4$ is observed (Figure 5), although it is less pronounced than with (*R*)-$BH_4$.

The minor influence of cAMP-dependent phosphorylation on the $K_m$ for tyrosine with (*R*)-$BH_4$ and (*R*,*S*)-6-methyl-$PH_4$, which has been reported by a number of investigators at both pH 6 and pH 7 (Lloyd & Kaufman, 1975; Vulliet et al., 1980; Lazar et al., 1982), can also be seen in reactions using 6,6-dimethyl-$PH_4$ and (*R*,*S*)-6-ethyl-6-methyl-$PH_4$. Both give $K_m$ values that are only slightly lower than with (*R*,*S*)-6-methyl-$PH_4$ (all measured at close to saturating cofactor concentrations). Two unusual observations with respect to affinity for tyrosine were made with (*R*,*S*)-6-ethyl-$PH_4$ and (*R*,*S*)-6-phenyl-6-methyl-$PH_4$. The latter tetrahydropterin caused a $K_m$ with cAMP-dependent phosphorylated tyrosine hydroxylase that was at least twice as high as any other determination. By comparison with other results in Table I, it can be seen that this high value is not a general result of either a phenyl group or disubstitution alone [cf. (*R*,*S*)-6-phenyl-$PH_4$ and 6,6-dimethyl-$PH_4$]. It appears that the extra bulk in combination with the conformational inflexibility of the phenyl ring in 6-phenyl-6-methyl-$PH_4$ may interfere with the association of substrate. Another intriguing exception to the average was found with (*R*,*S*)-6-ethyl-$PH_4$ which although giving a typical $K_m$ for tyrosine with unphosphorylated enzyme, showed a 3-fold drop upon cAMP-dependent phosphorylation to a value at least as low as with (*R*)-$BH_4$ and possibly considerably lower if the influence of substrate inhibition with the natural cofactor could be factored out.

Vigny and Henry (1982) have proposed that the effect of phosphorylation or polyanions is to neutralize a positive charge that is very near the catalytic site and whose electric field exerts a direct influence on cofactor binding and maximum velocity. Abate and Joh (1987) suggest that phosphorylation near the active site increases the rate of cofactor association. The structure–function relationships presented in Table II indicate a somewhat different view of cofactor binding that is represented by the model depicted in Figure 6.

In both unphosphorylated and cAMP-dependent phosphorylated tyrosine hydroxylase, the region into which the C6 side-chain projects is surrounded on one side by a nonpolar zone and oppositely by one or perhaps two groups responsible for recognition of the vicinal hydroxyls. A similar combination of nonpolar and hydrogen-bonding interactions has been observed in the X-ray crystallographic data for the binding domains of several carbohydrate moieties (Goldsmith & Fletterich, 1983). With tyrosine hydroxylase it is proposed that in the low-affinity state the hydrophobic and hydrophilic residues are removed from optimal interaction with the side chain. Upon phosphorylation by cAMP-dependent protein kinase, these residues collapse into positions that allow full involvement with the hydroxyl groups and the hydrocarbon backbone simultaneously. The specificity of the pocket increases as indicated by the altered response to disubstituted cofactors. Since changes in affinity can be correlated with side-chain structure, it seems likely that the primary effect of phosphorylation is to promote additional forces between cofactor and hydroxylase rather than removing a blockade limiting access to the catalytic site. The details of the interactions with the pterin ring are not yet known, but overall they appear also to be strengthened upon cAMP-dependent phosphorylation, since the $K_m$ for simple (*R*,*S*)-6-methyl-$PH_4$ decreases by 50-fold.

The nonpolar zone is probably not much larger than is required to give maximal van der Waals contact with the three carbon atoms in the side chain of the natural cofactor, since little decrease in $K_m$ results in extension of bulk beyond an ethyl group with the phosphorylated enzyme. As an example, the single amino acid valine-45 provides this function in binding AMP to rabbit muscle glycogen phosphorylase (Sprang et al., 1987). The 100-fold drop in $K_m$ from 6-methyl-$PH_4$ to 6-cyclohexyl-$PH_4$ with phosphorylated tyrosine hydroxylase compared to only a 10-fold decrease for the same cofactors with unphosphorylated enzyme may reflect not only a greater opportunity for overlap with this zone but possibly also exclusion of water from the domain occupied by the 6-substituent. The activities at pH 6 of a series of 6-monosubstituted tetrahydropterins with tyrosine hydroxylase (and also phenylalanine and tryptophan hydroxylases) that includes even longer chain lengths and branched alkyls have been recently reported (Armarego et al., 1987; Bigham et al., 1987). Kinetic analysis of these larger molecules at neutral pH, and with both forms of the enzyme, may help further delimit the size, shape, and changes upon phosphorylation of this portion of the pteridine binding site.

X-ray crystallographic studies of glycogen phosphorylase show that upon phosphorylation of serine-14 (i.e., comparing phosphorylase *b* with phosphorylase *a*) ion pairs are formed between the phosphate group and two arginine residues. The resulting conformational changes are transmitted to the catalytic site over a distance of more than 30 Å to promote greater substrate association (Sprang et al., 1987). However, glycogen phosphorylase and tyrosine hydroxylase respond in opposite ways to removal of their regulatory regions.

Proteolytic cleavage of a small peptide containing the phosphorylation site from the N-terminus of phosphorylase leaves the enzyme in a state that requires AMP for activity, shows little interaction of one substrate or effector on the kinetics of another, and has low affinity for glycogen substrate (Graves et al., 1968). Mild proteolysis of tyrosine hydroxylase also can remove a small N-terminal region within which is located the serine residue that is phosphorylated by cAMP-dependent protein kinase (Abate et al., 1988). However, in contrast to phosphorylase, the proteolytically cleaved hydroxylase displays lowered cofactor $K_m$ values, similar to enzyme that has been phosphorylated (Kuczenski, 1973; Vigny & Henry, 1982; Abate et al., 1988). Thus, the mechanisms of regulation by phosphorylation appear to be different in these two enzymes. With unphosphorylated tyrosine hydroxylase, the N-terminus may deactivate the catalytic site by forcing it into a shape that does not allow simultaneous optimization of all potential cofactor binding forces. The effect of cAMP-dependent phosphorylation would be to break this link, allowing a more relaxed conformation of the catalytic site that can take advantage of interactions with the vicinal hydroxyl groups, the side-chain backbone, and with the pterin ring.

Even considering that proteolytic cleavage seems to mimic the effect of cAMP-dependent phosphorylation, several alternative modes of interaction between phosphoserine and the rest of the protein can be formulated. Figure 6 illustrates one possibility in which the N-terminal region is linked with and distorts the cofactor binding domain in unphosphorylated hydroxylase. This link might then be broken by the formation of a new and stronger salt link to the phosphate, thus allowing the catalytic site to relax. Cleavage of the N-terminus might then also produce a similar relaxation and lowered $K_m$ values.

A study of the properties of the phenylalanine and tryptophan hydroxylase cofactor binding site using cofactor analogues has also been completed.[2] Many parallel relationships can be seen, as might be anticipated from the considerable degree of sequence homology among these enzymes. The trend toward higher affinity (up to a point) with increasing side-chain hydrophobicity can be seen with all three enzymes. However, the kinetic patterns with phenylalanine and tryptophan hydroxylases,[2] as isolated, bear a closer resemblence to those of the cAMP-dependent phosphorylated tyrosine hydroxylase.

The shift in the relationship among the cofactor analogues following phosphorylation may provide a method for determining the state of phosphorylation in vivo. For example, the ratio of the $K_m$ values of (*R*,*S*)-6-cyclhexyl-$PH_4$ and (*R*,*S*)-6-ethyl-$PH_4$ relative to (*R*,*S*)-6-methyl-$PH_4$ which is 1:6.7:10, respectively, with unphosphorylated enzyme becomes 1:1.7:100 after phosphorylation by cAMP-dependent protein kinase. Shifts in the pattern of $V_{max}/K_m$ were also found. Thus, analogues can be chosen such that, while similar enough to allow nearly equal penetration of cells, a differential response by tyrosine hydroxylase can be anticipated, the character of which will depend on the extent and type of phosphorylation. Studies are currently in progress to extend the above relationships to additional modes of protein phosphorylation.

## References


Abate, C., & Joh, T. H. (1987) *Soc. Neurosci. Abstr. 13*, 805.

Abate, C., Smith, J. A., & Joh, T. H. (1988) *Biochem. Biophys. Res. Commun. 151*, 1446–1453.

Armarego, W. L. F., Taguchi, H., Cotton, R. G. H., Battiston, S., & Leong, L. (1987) *Eur. J. Med. Chem.—Chim. Ther. 22*, 283–291.

Ayling, J. E., & Bailey, S. W. (1983) in *Biochemical and Clinical Aspects of Pteridines* (Curtius, H. Ch., Pfleiderer, W., & Wachter, H., Eds.) Vol. 2, pp 147–163, de Gruyter, Berlin.

Ayling, J. E., & Bailey, S. W. (1984) *Ninth International Congress of Pharmacology*, 1467P, Macmillan Press Ltd., London.

Ayling, J. E., Bailey, S. W., & Dillard, S. B. (1986) in *Chemistry and Biology of Pteridines* (Cooper, B. A., & Whitehead, V. M., Eds.) pp 391–394, de Gruyter, Berlin.

Bailey, S. W., & Ayling, J. E. (1980) *Anal. Biochem. 107*, 156–164.
Bailey, S. W., & Ayling, J. E. (1983) *Biochemistry 22*, 1790–1798.
Bigham, E. C., Smith, G. K., Rheinhard, J. F., Mallory, W. R., Nicohol, C. A., & Morrison, R. W. (1987) *J. Med. Chem. 30*, 40–45.
Bullard, W. P., & Capson, T. L. (1983) *Mol. Pharmacol. 23*, 104–111.
Burkert, U., & Allinger, N. L. (1982) *Molecular Mechanics*, ACS Monograph 177, American Chemical Society, Washington, DC.
Campbell, D. G., Hardie, D. G., & Vulliet, P. R. (1986) *J. Biol. Chem. 261*, 10489–10492.
Ellenbogen, L., Taylor, R. J., & Brundage, G. B. (1965) *Biochem. Biophys. Res. Commun. 19*, 708–715.
Fisher, D., & Kaufman, S. (1972) *J. Neurochem. 19*, 1359–1365.
Goldsmith, E., & Fletterick, R. J. (1983) *Pure Appl. Chem. 55*, 577–588.
Graves, D. J., Scharfenberg-Mann, S. A., Philip, G., & Oliveira, R. J. (1968) *J. Biol. Chem. 243*, 6090–6098.
Grima, B., Lamouroux, A., Boni, C., Julien, J. F., Javoy-Agid, F., & Mallet, J. (1987) *Nature (London) 326*, 707–711.
Hope, P. L., Cady, E. B., Chu, A., Delpy, D. T., Gardiner, R. M., & Reynolds, E. O. R. (1987) *J. Neurochem. 49*, 75–82.
Horvath, C., Melander, W., & Molnar, I. (1977) *Anal. Chem. 49*, 142–154.
Kaneda, N., Kobayashi, K., Ichinose, H., Kishi, F., Nakazawa, A., Kurosawa, Y., Fujita, K., & Nagatsu, T. (1987) *Biochem. Biophys. Res. Commun. 146*, 971–975.
Kapatos, G. (1987) *Soc. Neurosci. Abstr. 13*, 1180.
Kato, T., Oka, K., Nagatsu, T., Sugimoto, T., & Matsuura, S. (1980) *Biochim. Biophys. Acta 612*, 226–232.
Kaufman, S. (1975) in *Neurobiological Mechanisms of Adaptation and Behavior* (Mandell, A. J., Ed.) pp 127–136, Raven, New York.
Kaufman, S. (1987) *Enzymes (3rd Ed.) 18*, 217–282.
Kaufman, S., & Kaufman, E. E. (1985) in *Folates and Pterins* (Blakley, R. L., & Benkovic, S. J., Eds.) Vol. 2, pp 353–382, Wiley-Interscience, New York.
Kuczenski, R. (1973) *J. Biol. Chem. 248*, 2261–2265.
Lazar, M. A., Lockfeld, A. J., Truscott, R. J. W., & Barchas, J. D. (1982) *J. Neurochem. 39*, 409–422.
Lazar, M. A., Raese, J. D., & Barchas, J. D. (1983) *Neurochem. Int. 5*, 107–111.
Lloyd, T., & Kaufman, S. (1975) *Biochem. Biophys. Res. Commun. 66*, 907–913.
Lovenberg, W., Bruckwick, E. A., & Hanbauer, I. (1975) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2955–2958.
Markey, K. A., Kondo, S., Shenkman, L., & Goldstein, M. (1980) *Mol. Pharmacol. 17*, 79–85.
Miller, L. P., & Lovenberg, W. (1985) *Neurochem. Int. 7*, 689–697.
Nagatsu, T., Mizutani, K., Nagatsu, I., Matsuura, S., & Sugimoto, T. (1972) *Biochem. Pharmacol. 21*, 1945–1953.
Nelson, T. J., & Kaufman, S. (1987) *J. Biol. Chem. 262*, 16470–16475.
Numata, Y., Kato, T., Nagatsu, T., Sugimoto, T., & Matsuura, S. (1977) *Biochim. Biophys. Acta 480*, 104–112.
Oka, K., Ashiba, G., Sugimoto, T., Sadao, M., & Nagatsu, T. (1982) *Biochim. Biophys. Acta 706*, 188–196.
O'Malley, K. L., Anhalt, M. J., Martin, B. M., Kalsoe, J. R., Winfield, S. L., & Ginns, E. I. (1987) *Biochemistry 26*, 6910–6914.
Pfleiderer, W., & Mengel, R. (1971) *Chem. Ber. 104*, 2293–2312.
Pollock, R. J., Kapatos, G., & Kaufman, S. (1981) *J. Neurochem. 37*, 855–860.
Pradhan, S., Alphs, L., & Lovenberg, W. (1981) *Neuropharmacology 20*, 149–154.
Sadek, P. C., Carr, P. W., Doherty, R. M., Kamlet, M. J., Taft, R. W., & Abraham, M. H. (1985) *Anal. Chem. 57*, 2971–2978.
Segal, I. H. (1975) *Enzyme Kinetics*, Wiley, New York.
Shiman, R., Akino, M., & Kaufman, S. (1971) *J. Biol. Chem. 246*, 1330–1340.
Sprang, S., Goldsmith, E., & Fletterick, R. (1987) *Science (Washington, D.C.) 237*, 1012–1019.
Sze, P., Alderson, R. F., & Hedrick, B. J. (1983) *Brain Res. 268*, 129–137.
Tchapla, A., Heron, S., Colin, H., & Guiochon, G. (1988) *Anal. Chem. 60*, 1443–1448.
Vigny, A., & Henry, J. P. (1982) *Biochem. Biophys. Res. Commun. 106*, 1–7.
Vulliet, P. R., Langan, T. A., & Weiner, N. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 92–96.
Yamauchi, T., & Fujisawa, H. (1979) *J. Biol. Chem. 254*, 6408–6413.