Modulation by pterins of the phosphorylation and phenylalanine activation of phenylalanine 4-mono-oxygenase

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The interaction between phenylalanine 4-mono-oxygenase and analogues of the natural cofactor (6*R*)-tetrahydrobiopterin [(6*R*)-BH₄] was studied. The rate of cyclic AMP-dependent phosphorylation of phenylalanine 4-mono-oxygenase was inhibited only by those pterins [(6*R*)-BH₄, (6*S*)-BH₄ and 7,8-dihydrobiopterin (BH₂)] that were able to decrease the potency and efficiency of phenylalanine as an allosteric activator of the hydroxylase. Since BH₂ lacks cofactor activity, this was not required to modulate either the phosphorylation or the phenylalanine-activation of the hydroxylase. Half-maximal inhibition of the phosphorylation was observed at 1.9 μM-(6*R*)-BH₄, 9 μM-(6*S*)-BH₄ and 17 μM-BH₂. Competition experiments indicated that all three pterins acted through binding to the cofactor site of the hydroxylase. Since the phosphorylation site and the cofactor binding site are known to reside, respectively, in the *N*- and *C*-terminal domains of the hydroxylase, the pterins were able to induce an interdomain conformational change. BH₂, whose dihydroxypropyl group is not subject to epimerization, and (6*S*)-BH₄ both inhibited the phosphorylation less efficiently than did the (6*R*)-epimer of BH₄. Pterins with different spatial arrangements of the dihydroxypropyl side chain thus appeared to elicit different conformations of the phosphorylation site. The hydroxylase reaction showed a higher apparent *K_m* for (6*S*)-BH₄ than for (6*R*)-BH₄ both when the native and the phenylalanine-activated enzyme were tested. For the activated enzyme *V_{max}* was 40% lower with the (6*S*)-epimer than the (6*R*)-epimer, also when the more rapid enzyme inactivation occurring with the former cofactor was taken into account.

INTRODUCTION

Phenylalanine 4-mono-oxygenase (phenylalanine hydroxylase, EC 1.14.16.1) catalyses the formation of L-tyrosine from L-phenylalanine with an absolute requirement for a tetrahydropterin cofactor (Kaufman, 1971; Hasegawa *et al.*, 1979). The natural cofactor is (6*R*)-tetrahydrobiopterin [(6*R*)-BH₄], but a number of synthetic tetrahydropterins like (6*S*)-BH₄, 6,7-dimethyl-tetrahydropterin (DMPH₄), and tetrahydroneopterin can function as cofactors *in vitro* (Kaufman & Levenberg, 1959; Kaufman, 1962; Osanai & Rembold, 1971; Bailey & Ayling, 1978).

The hydroxylase catalyses the first step in the enzymic catabolism of phenylalanine in mammals and is subject to allosteric control (Nielsen, 1969; Shiman *et al.*, 1982) as well as control by covalent modification (Abita *et al.*, 1976; Fisher & Pogson, 1984). Both the substrate phenylalanine and the cofactor BH₄ affect the phosphorylatability of the hydroxylase by cyclic AMP-dependent protein kinase (Døskeland *et al.*, 1983, 1984a; Phillips & Kaufman, 1984).

The affinity of pterins for the hydroxylase is commonly estimated by their potency as cofactors or competitive inhibitors of cofactors in the hydroxylase reaction (Bailey & Ayling, 1983). The present detailed study of the effect of selected pterins on the phosphoryl-

atability of the hydroxylase was undertaken to have a measure of the affinities of the pterins for the native enzyme, undisturbed by phenylalanine and cofactor turnover. Using the phosphorylatability of the hydroxylase as a probe of its conformational state we also wanted to know whether one or more conformations could be induced by ligation of the cofactor site, whether cofactor activity of the ligand was required to bring about the conformational change, and whether ability of a pterin to modulate phosphorylatability was coupled with ability to affect phenylalanine binding to the activator site and interfere with the activating effect of that amino acid.

EXPERIMENTAL

Materials

L-Phenyl[2,3-³H]alanine (29 Ci/mmol) and [γ-³²P]ATP were from The Radiochemical Centre, Amersham, U.K. ATP, crystalline catalase, mixed histones (type IIS), and synthetic phosphate acceptor heptapeptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) were from Sigma Chemical Co. DMPH₄ was purchased from Aldrich Chemical Co. L-erythro-7,8-Dihydrobiopterin (BH₂), L-erythro-bi-pterin, and (6*R,S*)-D-erythro-5,6,7,8-tetrahydroneopterin were from Dr. B. Schircks Laboratories, Jona, Switzer-

Abbreviations used: *K_i*, apparent inhibition constant for ligands negatively modulating the phosphorylation of substrate, i.e. the concentration of ligand half-maximally inhibiting the phosphorylation; *K_d*, apparent equilibrium binding dissociation constant; DTT, dithiothreitol; BH₄, L-erythro-5,6,7,8-tetrahydrobiopterin; BH₂, L-erythro-7,8-dihydrobiopterin; DMPH₄, 6,7-dimethyl-5,6,7,8-tetrahydropterin.

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land. They were dissolved in aqueous solution containing 10 mM-HCl and 20 mM-DTT just before use in kinetic experiments. (6*R,S*)-BH₄ was prepared by catalytic reduction of biopterin, the diastereomers (6*R*)-BH₄ and (6*S*)-BH₄ were isolated (Bailey & Ayling, 1978; Haavik & Flatmark, 1983) in 30 mM-ammonium acetate, pH 3.5, and kept in liquid N₂ until used.

The concentration of DMPH₄ was determined at 297 nm and pH 7.0, using an absorption coefficient of 8.23 mM⁻¹·cm⁻¹. This value was estimated by titration of the pterin with 2,6-dichloroindophenol at pH 7.0, using the value 18.8 mM⁻¹·cm⁻¹ at 600 nm for the dye as determined by titration with ascorbate at pH 7.0. The concentration of BH₂ was determined spectrophotometrically at pH 6.8, using the absorption coefficient of 6.2 mM⁻¹·cm⁻¹ at 330 nm (Fukushima & Akino, 1968). The concentration of BH₄ was determined at pH 3.5, based on absorption coefficients of 12.2 (265 nm) for the (6*R*)-epimer and 12.6 (266 nm) for the (6*S*)-epimer (Matsuura *et al.*, 1980).

Preparation of enzymes

The hydroxylase was purified from rat liver by a slight modification (Døskeland *et al.*, 1982) of the procedure of Shiman *et al.* (1979). The concentration of the purified enzyme was estimated by assuming an absorbance of 1.0 at 280 nm (1 cm light path) for 1 mg/ml of enzyme solution (Shiman *et al.*, 1979). The concentration of the hydroxylase given refers to the amount of enzyme subunits.

The catalytic subunit of cyclic AMP-dependent protein kinase was prepared from the type II holoenzyme from bovine heart according to Øgreid & Døskeland (1981).

Assay of phenylalanine 4-mono-oxygenase activity

The hydroxylase activity was determined as described (Døskeland *et al.*, 1984a), except that the concentrations of Hepes/NaOH (180 mM) and DTT (4 mM) were higher. The high concentration of DTT was present to protect the cofactor against autooxidation and to protect the enzyme against inactivation in the presence of (6*S*)-BH₄ (Parniak & Kaufman, 1983). The high concentration of Hepes was required to buffer the ammonium acetate or HCl carried over with some cofactor solutions.

Phosphorylation assay

The phosphorylation of the hydroxylase was assayed at 30 °C in a reaction mixture containing 180 mM-Hepes/NaOH (pH 7.0), 0.1 mM-EGTA, 0.03 mM-EDTA, 4 mM-DTT, 10 mM-magnesium acetate, 60 μM-[γ-³²P]-ATP (2 Ci/ml), and 25 nM catalytic subunit of cyclic AMP-dependent protein kinase. The amount of phosphate incorporated into substrate was determined by spotting aliquots of the incubate on phosphocellulose strips (Roskoski, 1983). Since ammonium acetate, carried over with some pterin solutions, inhibited the rate of phosphorylation, care was taken to keep its concentration constant in each series of incubations.

Determination of kinetic constants

The apparent *K_i* for ligands (BH₂, BH₄) inhibiting the rate of hydroxylase phosphorylation was determined from Hill plots of the concentration-dependence of the ligand effect (Fig. 1b). The apparent *K_m* for cofactor in the hydroxylation reaction was determined from Hill

plots of the concentration dependence of the cofactor effect (Fig. 5). The Hill coefficient is the slope of the Hill plot at the point corresponding to an ordinate = 0. The values are based on the total concentrations of pterin (free + bound) in the incubations. Assuming that there is one pterin-binding site per monomer of hydroxylase, that all the enzyme is able to bind pterin, and that half of the binding sites are occupied at the pterin concentrations half-maximally inhibiting the phosphorylation rate, the *K_i* values calculated from the experiments of Figs. 1 and 2 should be decreased by 0.5 μM and 2 μM, respectively. The estimates of *K_m* should be decreased by 0.15 μM. It is possible that some of the enzyme prepared by the method of Shiman *et al.* (1979) is inactive (Gottschall *et al.*, 1982). This may explain the substoichiometric incorporation of phosphate obtainable in the phosphorylation reaction (Phillips & Kaufman, 1984; Døskeland *et al.*, 1984a), and raises the possibility that not all the enzyme may bind pterin. Because of this uncertainty the relatively modest error introduced by ignoring the difference between free and total pterin was not corrected for.

RESULTS

Modulation of enzyme phosphorylation by selected pterins

Tetrahydroneopterin and biopterin did not affect the rate of phosphorylation of phenylalanine hydroxylase by the catalytic subunit of cyclic AMP-dependent protein kinase at the concentrations of pterin (up to 0.2 mM) tested. The rate of phosphorylation decreased in a concentration dependent manner in the presence of (6*R*)-BH₄, (6*S*)-BH₄, or BH₂, the apparent *K_i* values being 1.9 μM, 9 μM (Fig. 1a) and 17 μM (Fig. 2), respectively. The Hill coefficient was 1.0 for either epimer of BH₄ (Fig. 1b), indicating nonco-operative binding to a single site. The effects appeared to be exerted via the substrate (hydroxylase) rather than via the kinase, since the phosphorylation of a synthetic heptapeptide or mixed histones was unaffected by BH₄ or BH₂. The possibility that the effect of BH₂ might simply be a consequence of the pterin being reduced in the incubation mixture by DTT, which is the case for its quinonoid form (Bublitz, 1977), was excluded by h.p.l.c. analyses of a mixture of BH₂ and DTT. Furthermore, BH₂ was chromatographically free from BH₄.

The phosphorylation rate could be more strongly inhibited by (6*R*)-BH₄ (about 70%) than by (6*S*)-BH₄ or BH₂ (45–55%). Thus, the inhibition obtained with the highest concentration (3 mM) of BH₂ tested was less than with 15 or 38 μM-(6*R*)-BH₄ (Fig. 2). It could be argued that the inhibition by BH₂ was less complete because the concentrations used were insufficient to saturate completely the cofactor-binding site. If this were the case the phosphorylatability of the hydroxylase incubated with moderate concentrations of (6*R*)-BH₄ should decrease further upon addition of BH₂. On the contrary, BH₂ partly relieved the inhibition brought about by 15 or 38 μM-(6*R*)-BH₄ (Fig. 2). This means that hydroxylase saturated with BH₂ differs in phosphorylatability from enzyme saturated with (6*R*)-BH₄. The facts that more BH₂ was required to overcome partially the effect of the natural cofactor at 38 μM than 15 μM, and that BH₂ was without effect in the presence of excess (6*R*)-BH₄ (Fig. 2),

Fig. 1. Effect of the two diastereoisomers of tetrahydrobiopterin on the cyclic AMP-dependent phosphorylation of phenylalanine 4-mono-oxygenase

(a) Shows the rate of phosphorylation (relative to that in the absence of added effector) as a function of the concentration of (6R)-BH₄ (○) and (6S)-BH₄ (●). The concentration of hydroxylase subunits was 1 μ M. For other details, see the Experimental section. (b) Represents Hill plots of the data shown in (a). $Y = v_0 - v_x / v_0 - v_{\min}$ is the fractional decrease of phosphorylation seen at the concentration x of the ligand, v_0 is the rate in the absence of ligand and $v_{\min}$ is the rate approached at very high concentration of ligand. The concentrations of effector given on the abscissa refer to the total (bound + free) amount of effector.

suggested that both pterins acted through a common site. This suggestion was further supported by the finding that 0.2 mM of the synthetic cofactor DMPH₄ abolished the effect of low concentrations of (6R)-BH₄, (6S)-BH₄, and BH₂ (results not shown).

The experiment shown in Fig. 3 represents an attempt to decide whether the effect of cofactor on hydroxylase phosphorylation was due to an increased K_m of the kinase for the hydroxylase or a decreased $V_{\max}$. The relative inhibition by (6R)-BH₄ was similar at all concentrations of enzyme (0.13–16 μ M) tested (Fig. 3), suggesting that the cofactor decreased the $V_{\max}$ of the kinase reaction. The phenylalanine-activated enzyme was phosphorylated at a higher rate than the native form at all enzyme concentrations tested (Fig. 3), suggesting that also phenylalanine affected the $V_{\max}$, rather than the K_m of the kinase for the hydroxylase. The data indicated a K_m of 20 μ M for the hydroxylase. This is somewhat lower

Fig. 2. Rate of phosphorylation of phenylalanine 4-mono-oxygenase as a function of the concentration of BH₂

The initial rate of phosphorylation was determined at various concentrations of BH₂ in the absence of (6R)-BH₄ (○) or added simultaneously with 15 μ M (●), 38 μ M (Δ), or 500 μ M (□) of (6R)-BH₄. The concentration of hydroxylase subunits was 4 μ M.

than the value (125 μ M) found by Phillips & Kaufman (1984), but considerably higher than that (2 μ M) reported by Wretborn *et al.* (1980).

The pterins were tested also for their ability to modulate the rate of phosphorylation of hydroxylase that had been preincubated (activated) by 10 mM-phenylalanine (in 180 mM-Hepes buffer, pH 7.0) for 10 min at 30 °C. (6R)-BH₄ decreased by up to 25% the rate of phosphorylation of the activated enzyme, half-maximal inhibition requiring about 8 μ M of the cofactor. The presence of (6S)-BH₄ (at 0.6 mM) or BH₂ (at 3 mM) gave only a 10–15% reduction in the rate of phosphorylation. Thus, (6S)-BH₄ and BH₂ were less efficient inhibitors than (6R)-BH₄ also of the phosphorylation of the activated form of the hydroxylase.

Effect of pterins on the activatability of phenylalanine hydroxylase by phenylalanine

BH₂ was able to decrease the potency of phenylalanine as an activator (Fig. 4). (6S)-BH₄ also decreased the potency of phenylalanine as an activator, but an exact determination of the degree of the shift in potency was difficult for this compound because some phenylalanine was converted to tyrosine, and because some irreversible enzyme inactivation occurred (see Fig. 7 and the last paragraph of this section). We have previously demonstrated a thermodynamic coupling between ligand binding to the allosteric activator site for phenylalanine and BH₄ binding to the cofactor site, BH₄ [i.e. a mixture of 70% (6R)- and 30% (6S)-epimer was used] decreasing the potency of phenylalanine as an activator (Døskeland *et al.*, 1984a). It can thus be concluded that binding of

Fig. 3. Effect of phenylalanine 4-mono-oxygenase concentration on the phosphorylation rate

The initial rate of phosphorylation (v) of the hydroxylase by 5 nM of catalytic (C) subunit was measured in incubations containing 50 μM - BH_4 ($\bullet$), 0.7 mM-phenylalanine (Δ) or none of the above ($\circ$). The concentration of hydroxylase was varied through a 128-fold range. The rate (v) is expressed as mol of ^{32}P incorporated into the hydroxylase/min per mol of hydroxylase subunit. The V_{max} was calculated to be 250 in the absence of effectors (phenylalanine, BH_4). The K_m for the hydroxylase was about 20 μM whether phenylalanine or BH_4 was present or not.

BH_2 as well as of either epimer of BH_4 decreased the potency of phenylalanine as an activator of the hydroxylase.

The experiment shown in Table 1 was set up to test the effect of pterins on the efficiency rather than potency of phenylalanine as an activator. The hydroxylase was briefly preincubated with various pterins, and thereafter with 5 mM unlabelled phenylalanine. This high concentration of phenylalanine would appear sufficient to saturate the allosteric phenylalanine site both in the absence and presence of pterins (Shiman, 1980; Kaufman & Mason, 1982; Døskeland *et al.*, 1984a; Fig. 4). The degree to which the pterin had inhibited the activating effect of 5 mM-phenylalanine was assessed by measuring the formation of labelled tyrosine from added [^3H]phenylalanine in the presence of excess (0.2 mM) (6*R*)- BH_4 . This experimental design allowed the testing of pterins without cofactor activity (like BH_2). The results (Table 1) showed that those pterins already found to be negative modulators of the hydroxylase phosphoryl-

Fig. 4. Potency of phenylalanine as an activator of phenylalanine 4-mono-oxygenase in the presence of BH_2

Hydroxylase (1 μM) was preincubated for 5 min at 25 $^{\circ}\text{C}$ with various concentrations (see the abscissa) of phenylalanine, and either no pterin ($\circ$), 40 μM - BH_2 ($\bullet$), or 80 μM - BH_2 ($\square$). The incubation was started by the addition of (6*R*)- BH_4 to a final concentration of 170 μM together with [^3H]phenylalanine. The final concentration of phenylalanine was 1 mM in all the incubation media. After 2 min of incubation, the amount of tyrosine formed was determined as described in Experimental section.

Table 1. Effect of preincubation with selected pterins on phenylalanine activation of phenylalanine 4-mono-oxygenase

Phenylalanine hydroxylase (1.1 μM) was preincubated for 0.5 min at 25 $^{\circ}\text{C}$ in assay buffer without phenylalanine and with 10 mM-DTT. The concentrations of pterins were as indicated. Thereafter phenylalanine (50 mM in 180 mM-Hepes buffer, pH 7.0) was added to a final concentration of 5 mM, and 20 s later the hydroxylase assay was started by the addition of 4 vol. of assay buffer with [^3H]phenylalanine (5 $\mu\text{Ci}/\text{ml}$) and (6*R*)- BH_4 (250 μM), and the incubation (0.25 ml) was allowed to proceed for 2 min. Formed [^3H]tyrosine was determined as described in the Experimental section. The values in the right-hand column are given in units relative to the activity in the absence of pterin (12 nmol of tyrosine/2 min of incubation).

Pterin	Concentration (μM) during preincubation	Tyrosine formed (relative units)
None	—	1.0
BH_2	80	0.54
Tetrahydroneopterin	200	1.1
DMPH $_4$	200	0.94
(6 <i>R</i>)- BH_4	6	0.51
	12	0.36
	100	0.12
(6 <i>S</i>)- BH_4	12	0.96
	25	0.69
	100	0.46

ation, i.e. (6*R*)- BH_4 , (6*S*)- BH_4 , and BH_2 , also inhibited the activation of the enzyme by phenylalanine, whereas DMPH $_4$ and tetrahydroneopterin failed to affect both the phenylalanine-activation (Table 1) and the phosphoryl-

Fig. 5. Stereoselective effects of tetrahydrobiopterin diastereoisomers on the kinetics of the hydroxylation reaction with native phenylalanine 4-mono-oxygenase

The rates of tyrosine formation with (6*R*)-BH₄ (○) or (6*S*)-BH₄ (●) as the cofactor were measured fluorimetrically as described in the Experimental section. The reaction was started by the addition of the native (non-activated) enzyme at the final concentration of 0.3 μM. The concentration of ammonium acetate was 3 mM in all the incubations. A Hill plot of the data is shown,

ation (see the preceding paragraph). BH₂ and BH₄ thus decreased the efficiency (Table 1) as well as the potency (see above and Fig. 4) of phenylalanine as an activator of the hydroxylase.

Comparison of (6*R*)- and (6*S*)-BH₄ as cofactors for non-activated and activated hydroxylase

The hydroxylase activity of native (Fig. 5) and phenylalanine-activated (Fig. 6) hydroxylase was studied

as a function of the concentrations of (6*R*)-BH₄ and (6*S*)-BH₄. The slopes of the Hill plots for native (Fig. 5) and activated enzyme (not shown) were similar for either cofactor, with Hill coefficients close to unity (range 0.95–1.2). The apparent *K_m* was higher for the (6*S*)-epimer: 6.3 μM for native and 32 μM for activated enzyme, than for the (6*R*)-epimer: 2.5 μM for native and 18 μM for activated enzyme. The time course of the hydroxylase-catalysed tyrosine formation was compared using a near-saturating concentration (100 μM) of (6*R*)-BH₄ or (6*S*)-BH₄ as the cofactor. For non-activated enzyme the tyrosine accumulation was proportional to time up to 2 min, and declined only slowly thereafter (Fig. 7). Enzyme activated by preincubation with phenylalanine showed a more rapid time-dependent decrease of reaction rate (Fig. 7), the ratio between the hydroxylase activity with (6*S*)- and (6*R*)-BH₄ decreasing as a function of time. The first order nature of the decline in this ratio made it possible by extrapolation to estimate its value (0.6) at zero time of incubation. In order to rule out that the 40% lower activity with the (6*S*)-epimer was due to lack of saturation of the cofactor-binding site of the activated enzyme, it was tested whether the addition of increasing concentrations of (6*R*)-BH₄ to hydroxylase incubated with (6*S*)-BH₄ could easily increase the enzyme activity towards that with a moderately high concentration of the (6*R*)-epimer alone. It appeared that more (6*R*)-BH₄ was required to obtain maximal activity in the presence than absence of the (6*S*)-epimer (Fig. 6*b*, inset). This demonstrated that activated enzyme saturated with (6*S*)-BH₄ was a less efficient catalyst than enzyme saturated with the natural cofactor. On the other hand, the *V_{max}* of native (non-activated) enzyme was at least as high with the (6*S*)-epimer as with the (6*R*)-epimer as the cofactor (Fig. 7).

Fig. 6. Kinetics of the hydroxylation reaction with phenylalanine-activated enzyme

(a) The experimental conditions were as in Fig. 3, except that the enzyme had been preincubated for 5 min with phenylalanine (25 °C) before the reaction was started by cofactor, i.e. either (6*R*)-BH₄ (□) or (6*S*)-BH₄ (■). (b) The activity was measured at increasing concentration of (6*R*)-BH₄ in the absence (□) or presence (▲) of 70 μM-(6*S*)-BH₄.

Fig. 7. Time course of the hydroxylation reaction

The tyrosine formed by activated enzyme (□, ■) is shown in the upper two curves, and by native enzyme (○, ●) in the lower curve. The cofactor was either 100 μM-(6R)-BH₄ (○, □) or 100 μM-(6S)-BH₄ (●, ■). The straight broken lines show the initial rates of tyrosine formation by activated enzyme with (6R)-BH₄ (upper line) and (6S)-BH₄ (lower line) as the cofactor.

DISCUSSION

Phenylalanine 4-mono-oxygenase is a two-domain protein in which the C-terminal domain contains the catalytic site (Iwaki *et al.*, 1986). The N-terminal domain contains the serine residue (Wretborn *et al.*, 1980; Robson *et al.*, 1984; Iwaki *et al.*, 1985, 1986) which is subject to phosphorylation by cyclic AMP-dependent protein kinase (Abita *et al.*, 1976) and by Ca²⁺/calmodulin-dependent multifunctional protein kinase (Døskeland *et al.*, 1984b). Phosphorylation sensitizes the enzyme to activation by phenylalanine (Shiman *et al.*, 1982; Døskeland *et al.*, 1984a). By binding to an allosteric site distinct from the substrate binding site (Shiman, 1980; Kaufman & Mason, 1982; Døskeland *et al.*, 1984a), phenylalanine relieves the enzyme of the tonic inhibition exerted by the regulatory N-terminal fragment (Iwaki *et al.*, 1986). We have previously found that phenylalanine and a number of its analogues modulate the efficiency of the hydroxylase as a kinase substrate (Døskeland *et al.*, 1984a). Since it is not known where in the enzyme molecule the allosteric phenylalanine-binding site resides, it cannot be decided whether the effect of phenylalanine on phosphorylatability reflects an intra- or inter-domain conformational change. The cofactor binding site is in the C-terminal domain and any effect of this site on the phosphorylatability of the near N-terminally located serine therefore has to be inter-domain. In the present study, the natural cofactor (6R)-BH₄, as well as its synthetic (6S)-epimer (Fig. 1) and BH₂ (Fig. 2), were found to depress the phosphorylatability of the hydroxyl-

ase by cyclic AMP-dependent protein kinase. The concentrations of (6R)- and (6S)-BH₄ required for half-maximal modulation of the phosphorylation rate (1.9 and 9.0 μM, respectively; Fig. 1) were similar to those (2.5 and 6.3 μM, respectively; Fig. 5) required for half-maximal hydroxylase activity of the native (i.e. non-activated) enzyme. Furthermore, the Hill coefficients characterizing the interaction between the pterins and the hydroxylase were of similar magnitude (about 1), whether deduced from phosphorylation data (Fig. 1b) or data on tyrosine formation (Fig. 5). This suggests that the two diastereomers of BH₄ acted via the cofactor-binding subsite of the active site. In further support of this contention was the finding that the synthetic cofactor DMPH₄ was able to counteract competitively the effect of either epimer of BH₄ on the phosphorylation reaction (see the first paragraph of the Results section). The natural cofactor was not only more potent than (6S)-BH₄, it also gave a more profound inhibition of the phosphorylation rate (Fig. 1), explaining the observation (Phillips & Kaufman, 1984) that 8.6 μM of the (6S)-epimer inhibited the phosphorylation less than did 8.6 μM of the (6R)-epimer. Also, BH₂ was a less efficient inhibitor of the phosphorylation than (6R)-BH₄. The finding that BH₂ in a competitive manner could partly overcome the inhibition caused by (6R)-BH₄ (Fig. 2) showed that BH₂ modulated the phosphorylatability by acting through the same site as the natural cofactor. Thus it appears that different pterins, acting through the same site on the C-terminal domain of the hydroxylase, can give rise to more than one conformation of the region of the N-terminal domain containing the phosphorylation site. This is in contrast with the conformational change resulting from occupancy of the allosteric phenylalanine-binding site, where all ligands give the same effect on phosphorylatability (Døskeland *et al.*, 1984a).

Since BH₂ is without cofactor activity (Bailey & Ayling, 1983), such activity cannot be necessary for the induction of a conformational change. It has been postulated that cofactor (6-methyltetrahydropterin) reduces and activates the enzyme in addition to participating in the hydroxylation reaction, only the unoxidized (tetrahydro) form of the cofactor being active (Marota & Shiman, 1984; Wallick *et al.*, 1984). Since it is unlikely that BH₂ could reduce the enzyme, such enzyme reduction is probably not required to induce a conformational change. The data point to the hydroxypropyl side chain of the pterins as being essential for their ability to induce the conformational change. Replacement of the whole hydroxypropyl side chain by a methyl group (Phillips & Kaufman, 1984; Døskeland *et al.*, 1984a; the present paper) or the introduction, as in tetrahydroneopterin, of another hydroxyl group in position 3 of the side-chain (the present paper) completely abolished the ability of the pterin to affect the phosphorylation rate. Also the steric configuration of the hydroxypropyl chain is of importance. Thus, as already mentioned, the phosphorylatability in the presence of (6R)-BH₄ was different from that with either its (6S)-epimer or with BH₂, whose hydroxypropyl chain is free to rotate (Bieri, 1977; Schircks *et al.*, 1978). Since tetrahydroneopterin, if anything, is more bulky than BH₄, the effects of BH₄ and BH₂ can hardly be ascribed to steric hindrance of the phosphorylation reaction.

We have previously (Døskeland *et al.*, 1984a) shown that BH₄ (i.e. a mixture of the R- and S-epimers)

decreased the affinity of phenylalanine for its allosteric site. In the present study BH_2 was found to decrease the potency of phenylalanine as an activator (Fig. 4), showing that also the conformational change induced by BH_2 influences the allosteric phenylalanine-binding site. Furthermore, all the pterins that affected the rate of hydroxylase phosphorylation inhibited the activation of the enzyme by phenylalanine (Table 1), even at a concentration (5 mM) of the amino acid sufficiently high to saturate the allosteric site. This means that the conformational change induced by BH_4 as well as BH_2 affects the ability of the allosteric site to activate the hydroxylase, the affinity of that site for phenylalanine, and the phosphorylatability of the enzyme. The mechanistically most simple explanation of these diverse effects of pterin binding to the cofactor site is that this site, as well as the phosphorylation site and the allosteric phenylalanine-binding site, all are near the interface between the *N*- and *C*-terminal domains, and that the pterins affect the relative position of the two domains. The recent findings of Iwaki *et al.* (1986) that (6*R*)- BH_4 modulates the rate of chymotryptic cleavage in the region linking the two domains fits this possibility. The situation of the active site in a cleft between domains capable of ligand-dependent movement relative to each other is not uncommon: it has been noted for citrate synthetase, alcohol dehydrogenase and hexokinase (Bennett & Huber, 1984).

The affinity of ligands for the hydroxylase could be deduced from the concentration-dependence of their effect on hydroxylase phosphorylatability. The potency of (6*R*)- BH_4 as a modulator of phosphorylatability (apparent K_i 1.9 μM) in the absence of phenylalanine (Fig. 1) was similar to its potency (apparent K_m 2.5 μM) as a cofactor for the non-activated enzyme (Fig. 5). This contrasts sharply with the data of Phillips *et al.* (1984), who reported that the native enzyme in the absence of phenylalanine had a 100-fold higher affinity for BH_4 (apparent equilibrium dissociation constant K_d 0.02 μM) than found in the present study. The data of Phillips *et al.* (1984) were based on the quenching of intrinsic fluorescence, and the enzyme concentration was much higher than the apparent K_d . Although the reason for the discrepancy is not clear, the estimation of quantitative binding parameters for high-affinity interactions is very uncertain at such a high receptor concentration (Chang *et al.*, 1975). We have recently repeated the fluorescence-quenching experiments of Phillips *et al.* (1984) using a lower enzyme concentration, and although the data are less precise than those obtained from the phosphorylation experiments, they clearly suggest a K_d for (6*R*)- BH_4 above 0.5 μM (Haavik *et al.*, 1986). The (6*S*)-epimer of BH_4 was clearly less potent than the natural cofactor as a modulator of phosphorylation (Fig. 1), in apparent contrast with the reported near-equipotence of the epimers as cofactors for the phenylalanine-activated enzyme (Bailey & Ayling, 1978). We found the (6*S*)-epimer to be less potent than its natural congener also in the hydroxylase assay (Figs. 5 and 6), but the difference in potency was less marked than in the phosphorylation assay. It should be noted that the hydroxylase was subject to a time-dependent inactivation during the assay with (6*S*)- BH_4 (Fig. 7). Such inactivation may result from products of (6*S*)- BH_4 formed during the hydroxylase reaction, especially in the absence of reducing agents (Parniak & Kaufman, 1983). The use of

activated enzyme in the absence of DTT (Bailey & Ayling, 1978) would maximize the formation from the (6*S*)-epimer of substances that interact with and modify the hydroxylase. Since the phosphorylation assay is carried out in the absence of the phenylalanine substrate it should be less affected by this problem, and therefore give a more true estimate of the cofactor affinity.

The present study has been concerned with mechanistic aspects of the interaction between cofactor analogues and the hydroxylase *in vitro*. It is not known to what extent variation of the level of BH_4 may control the activation by phenylalanine or the phosphorylation of the hydroxylase *in vivo*. The recent demonstration that the concentration of BH_4 is affected by hormones and cyclic AMP in some cell types (Duch *et al.*, 1986) raises this possibility.

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