The Effect of Ligands of Phenylalanine 4-Monoxygenase on the cAMP-dependent Phosphorylation of the Enzyme*

(Received for publication, January 25, 1984)

Anne P. Deskeland‡, Stein O. Deskeland‡, Dagfinn Ögreid, and TorgergeFlatmark‡

From the Departments of Biochemistry and Anatomy, The Preclinical Institutes, University of Bergen, Årstadtoven 19, N-5000 Bergen, Norway

The rate of phosphorylation of phenylalanine 4-monoxygenase by the cAMP-dependent protein kinase was found to be under substrate-directed regulation. Thus, L-phenylalanine made the hydroxylase a better substrate for the kinase whereas the cofactor L-erythro-5,6,7,8-tetrahydrobiopterin (BH₄) was lacking in its effect. The dephosphorylation of the enzyme by the kinase in the presence of high concentrations of MgADP was also stimulated by phenylalanine and inhibited by BH₄.

A kinetic analysis indicated that the effects of phenylalanine and BH₄ were mediated by distinct sites coupled by a free energy of 3.2 kJ mol⁻¹.

Among the ligands tested, only phenylalanine and BH₄ affected the phosphorylation of the hydroxylase at physiologically relevant concentrations. Whereas higher concentrations of several naturally occurring or synthetic amino acids acted like phenylalanine, the widely used synthetic cofactor 6,7-dimethyltetrahydrobiopterin did not mimic the effect of BH₄.

Less phenylalanine was required to activate the phosphorylated hydroxylase (0.9 mol of phosphate/subunit) than the dephosphorylated enzyme (0.07 mol of phosphate/subunit). This was true whether BH₄ was present or not.

In conclusion, the substrate phenylalanine makes the hydroxylase more prone to cAMP-dependent phosphorylation, which in turn sensitizes the enzyme towards allosteric activation by phenylalanine. The joint operation of these mechanisms in vivo would increase the efficiency with which phenylalanine controls the activity of the enzyme.

Phenylalanine 4-monoxygenase (phenylalanine hydroxylase; EC 1.14.16.1) catalyzes the hydroxylation of phenylalanine to tyrosine with the L-erythro-5,6,7,8-tetrahydrobiopterin as the natural cofactor (1, 2). Since the reaction is irreversible (1), the activity of the enzyme must be carefully controlled to avoid depletion of the body stores of phenylalanine, an essential amino acid.

Phenylalanine can activate the hydroxylase¹ (3, 4), presumably by binding to an allosteric site which is distinct from the substrate-binding site (5, 6). The hydroxylase is also a substrate for cAMP-dependent phosphorylation in vitro (7, 8) and in vivo (9, 10), and the stimulation of the hydroxylase activity by this covalent modification (7, 11) seems at least in part to be due to sensitization of the enzyme towards activation by phenylalanine (12, 13, this study).

The primary aim of the present study was to learn whether ligands of the hydroxylase, like phenylalanine, could modulate the cAMP-dependent phosphorylation of the enzyme. It was of particular interest to know if such ligand-modulated phosphorylation was likely to occur under physiologically relevant conditions, and if it might enhance the fine control of the hydroxylase activity by phenylalanine. A second aim was to characterize the specificity of the sites mediating the ligand effects as well as any interactions between such sites. Finally, the effect of phenylalanine and BH₄ on the kinase-catalyzed dephosphorylation of the phosphenzyme was studied.

**EXPERIMENTAL PROCEDURES**

Materials—[γ-³²P]ATP (4000 Ci/mmol) and L-phenyl-[2,3-³H]alanine (29 Ci/mmol) were from the Radiochemical Centre, Amersham. ATP, ADP, the synthetic Ser peptide (kemptide), histone (type II A), catalase, and all amino acids and their analogs were from Sigma. 6,7-Dimethyltetrahydropterin was from Aldrich (Steinheim, FGR).

Tetrahydrobiopterin was generously donated by Hoffman-LaRoche (Basel, Switzerland). The compound was dissolved in 5 mM HCl and kept at −20 °C under N₂. The particular batch used contained 70% of the R-diastereoisomer and 30% of the S-diastereoisomer, as analyzed by high-performance liquid chromatography according to Bailey and Ayling (14). The concentrations of the cofactors given refer to that of the R-diastereoisomer.

The ³H-labeled phenylalanine was further purified by high-performance liquid chromatography using a 50 × 0.3 cm column packed with Zipax SCX (du Pont Chemical Co.) and 10 mM sodium acetate (pH 3.9) containing 2% (v/v) ethanol as the mobile phase. The ³H-labeled phenylalanine (³H₀ = 3 min at a flow rate of 2 ml/min) was in this way separated from a radioactive contaminant having the same retention time as tyrosine (³H₀ = 0.9 min).

Phenylalanine 4-monoxygenase was prepared from rat liver by a slight modification (15) of the procedure of Shiman et al. (16). The preparation of enzyme used contained 0.07 mol of phosphate per subunit of Mₛ = 51,000 and had a specific activity of 1300 nmol min⁻¹ mg⁻¹ with 6,7-dimethyltetrahydropterin as the cofactor. The phosphate content was assayed essentially as described by Stull and Buss (17). The concentrations of the hydroxylase given refer to the amount of enzyme subunits.

CAMP-dependent protein kinase was either the type I holoenzyme from rabbit skeletal muscle (8) or the C subunit of the type II holoenzyme from bovine heart prepared as described in Ref. 18 or received as a gift from Dr. J. D. Corbin, Howard Hughes Medical Institute, Nashville, TN. The concentrations of kinase given in the text refer to the amount of C subunit.

**Assay of cAMP-dependent Phosphorylation**—The standard protein kinase reaction mixture contained 15 mM Hepes·NaOH (pH 7.0), 0.1 mM ethylene glycol bis(β-aminoethylether)-N,N',N''-tetraacetic acid (EGTA), 80 μg of calf intestinal alkaline phosphatase, and 0.2–1.0 mg of protein in a final volume of 0.1 ml. The reaction was initiated by adding the enzyme and was allowed to proceed at 30 °C for 5 or 30 min with constant shaking. Reaction was terminated by adding an equal volume of 3% (w/v) trichloroacetic acid (TCA) to precipitate the protein. To determine the amount of substrate phosphate associated with the enzyme, the precipitate was washed four times with 5% TCA and then transferred to scintillation vials where the TCA was allowed to evaporate. The vials were then filled with 10 ml of Aquasol-2 (New England Nuclear) and counted in a liquid scintillation counter. The incubation mixture was also analyzed for the amount of substrate phosphate by the method of Berkley and MacGillivray (19). The unit of activity is defined as nmol of substrate phosphate per mg of protein per hour.

* This work was supported by the Norwegian Council for Research on Mental Retardation, The Nordic Insulin Foundation, and the Norwegian Cancer Society. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† The abbreviations used are: hydroxylase, phenylalanine monooxygenase; BH₄, 6R-diastereoisomer of L-erythro-5,6,7,8-tetrahydrobiopterin; kemptide, L-leucyl-L-arginyl-L-arginyl-L-alanyllL-seryl-L-leucylglycine; ³H₀, retention time; C subunit, catalytic subunit of cyclic AMP-dependent protein kinase; Kₛ and Kₗ, concentration of effector required for half-maximal enhancement and inhibition, respectively, of phosphorylation rate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

² Jan Haavik, personal communication.
acid, 0.03 mM EDTA, 3 mM dithiothreitol (Hepes buffer), 10 mM Mg
acetate and 20 mM [γ-32P]ATP (2 pCi/ml). Sometimes the incubation
contained one or more of the following: cAMP (2 μM), serum albumin
(0.5 mg/ml), histone (0.7 mg/ml), and kemptide (100 μM). The
concentrations of kinase and hydroxylase and other details are given
in the legends to the figures and Table I. The amount of 32P
transferred to the substrate was determined by spotting aliquots of the incubate
on filter discs (19, 20) or phoshocellulose strips (21).

Preparation of Phosphorylated Phenylalanine 4-Monoxygenase—
10 μM hydroxylase was incubated for 30 min at 30 °C under phosphory-
lation conditions (see above). The concentration of C subunit was
Fig. 6, was added to 112.5
pl of the supernatant were
incubated at a final concentration of 4 μM monomer in a phos-
phorylating system (see “Experimental Procedures”), containing 60
μM [γ-32P]ATP, and 25 nM of C subunit. The incubations contained

Fig. 1. The effect of phenylalanine and BH₄ on the time
course of the phosphorylation of phenylalanine 4-mono-
oxigenase by cAMP-dependent protein kinase. The hydroxylase
was incubated at a final concentration of 4 μM monomer in a phos-
phorylating system (see “Experimental Procedures”), containing 60
μM [γ-32P]ATP, and 25 nM of C subunit. The incubations contained
either no effector (O), 50 μM BH₄ (●), 1 mM phenylalanine (Δ), or a
combination (▲) of the two effectors (50 μM BH₄ + 1 mM phenyl-
alanine). At timed intervals, aliquots (45 μl) were removed for the assay of 32P incorporated.

Fig. 2. The effect of the addition of phenylalanine on the
time course of phosphorylation of phenylalanine 4-mono-
oxigenase. Two parallel incubations (1.2 ml) were performed, each
containing 4 μM hydroxylase subunit and 1 nM C subunit. Aliquots
(2 × 50 μl) were withdrawn during the first 4 min of incubation for
the assay of 32P incorporated into the enzyme. After 5 min, one of
the incubations (●) received 37 μl of 20 mM phenylalanine in assay
buffer and the control (O) 37 μl of assay buffer, and aliquots (2 ×
52.6 μl) were removed at timed intervals.

The rate of phosphorylation of phenylalanine 4-monoxygenase
increased up to 2-fold when assayed in the presence of several analogs
of phenylalanine⁵ (Table I). The synthetic analogs β-2-thienyl-
alanine and p-chlorophenylalanine were moderately less po-
tent, whereas the naturally occurring amino acids tested were
at least 2 orders of magnitude less potent than phenylalanine.
For all the analogs, the transition from no effect to full effect
on the phosphorylation occurred in a relatively narrow concen-
tration range (Table I and data not shown). The artificial
cofactor 6,7-dimethyltetrahydropterin, much used in studies
on the activity of the hydroxylase in vitro (24, 25), did not

³-L-Tyrosine at a nominal concentration of 5 mM induced a 1.5-
fold increase in the rate of phosphorylation of the hydroxylase. No
further increase was obtained by raising the concentration of tyrosine
beyond 5 mM, presumably because of the limited solubility of this
amino acid.

RESULTS

Phenylalanine and BH₄ Alter the Rate, but Not the Extent,
of the Phosphorylation of Phenylalanine 4-Monoxygenase—
The rate of phosphorylation of phenylalanine hydroxylase by
cAMP-dependent protein kinase increased in the presence of
phenylalanine and decreased in the presence of the natural
cofactor BH₄ (Fig. 1). The magnitude of the responses to these
effectors were independent of the source of the kinase used
(cAMP-dependent protein kinase holoenzyme type I or the C
subunit of type II kinase).

The initial rate of phosphorylation was linear with respect to
the concentration of kinase (range tested: 0.5–100 nM) and
time (Fig. 2). The onset of the effect of phenylalanine was
rapid as compared to the time scale of the experiments (Fig.
2).

The apparent end point of 32P incorporation into the
hydroxylase was not affected by substrate or cofactor (Fig. 1) or
the method used to estimate the incorporation of 32P, and the
values obtained ranged from 0.7 to 0.85 mol of 32P/mol
of hydroxylase subunit.

On the Specificity of the Effects of Ligands on the Phospho-
rylation of Phenylalanine 4-Monoxygenase—The rate of

5 32P incorporation was shifted the order of elution
from that of Bailey and Ayling (22) by using the ion-pair-forming
reagent tetrabutylammonium ion in the mobile phase. This modification
retained between 2.5 and 3 min. In samples lacking enzyme (blanks)
less than 0.02% of the applied radioactivity was eluted in  the fraction
where the hydroxylase was preincubated with both phenylala-
nine and BH₄. For samples of hydroxylase not  containing preformed
tyrosine, identical values of enzyme activity were obtained whether
the tyrosine, identical values of enzyme activity were obtained whether
the usual method or the method using the ion-pair-forming
reagent was used.

It should be noted that by measuring the hydroxylase activity as
the appearance of isotopically labeled tyrosine, interference from any
unlabeled tyrosine carried over from the phosphorylations was avoided.
This was especially important for those experiments (to be shown in
Fig. 6) where the hydroxylase was phosphorylated with both phenyl-
alanine and BH₄. For samples of hydroxylase not containing preformed
tyrosine, identical values of enzyme activity were obtained whether
formed tyrosine was detected by its fluorescence or its radioactivity.

Protein was measured by the method of Bradford (23) using bovine
serum albumin as the standard.
Effect of various compounds on the rate of phosphorylation of phenylalanine 4-monooxygenase

The initial rate of phosphorylation of phenylalanine 4-monooxygenase (2 μM with respect to subunits) was determined by analyzing duplicate aliquots removed after 4, 8, and 12 min of incubation for the amount of [γ-32P]ATP incorporated. The incubations contained 100 mM (rather than 15 mM) Hepes-NaOH (pH 7.0), 60 μM [γ-32P]ATP, and 2 mM C subunit. The concentrations of effector given refer to the final concentrations in the incubations. The relative phosphorylation rate is the ratio between the rate in the presence and absence of effector (mean of three experiments, the range being less than ±15% of the values shown). Parallel incubations were run in which kemptide or histone replaced hydroxylase as the substrate and the concentration of kinase was 0.2 nM. Except for a 20% inhibition by 20 mM tryptophane, none of the compounds tested significantly affected the rate of phosphorylation of kemptide or histone.

| Compound added     | Concentration | Relative rate of phosphorylation |
|--------------------|---------------|----------------------------------|
| None               | 0.005         | 1                                |
| L-Phenylalanine    | 0.05          | 1.48                             |
|                    | 0.5           | 2.07                             |
|                    | 25            | 1.93                             |
| β-2-Thienyl-DL-alanine | 0.07       | 1.02                             |
|                    | 0.2           | 1.15                             |
|                    | 0.4           | 1.56                             |
|                    | 0.65          | 1.87                             |
|                    | 2.0           | 1.86                             |
| DL-p-Chlorophenylalanine | 0.7        | 0.98                             |
|                    | 1.8           | 1.46                             |
|                    | 3.6           | 1.98                             |
| L-Tryptophan       | 1.0           | 1.01                             |
|                    | 3.1           | 1.12                             |
|                    | 6.3           | 1.76                             |
|                    | 20            | 1.65 (2.08)*                     |
| L-Norleucine       | 5             | 1.02                             |
|                    | 12.5          | 1.71                             |
|                    | 25            | 1.97                             |
| L-Methionine       | 5             | 0.96                             |
|                    | 12.5          | 1.54                             |
|                    | 25            | 2.00                             |
| α-Phenylalanine    | 19            | 1.15                             |
| L-Alanine          | 1             | 1.08                             |
| 6,7-Dimethyltetrahydropterin | 25    | 1.09                             |
| BH4                | 0.4           | 1.18                             |
|                    | 0.003         | 0.70                             |
|                    | 0.03          | 0.51                             |

*The value in parentheses has been corrected for the assumedly nonspecific inhibition of phosphorylation by that amino acid as determined using kemptide as the substrate.

The concentration of BH4 was estimated to be 0.5 mg/g of rat liver (30) and that of BH4 to be 2.5-2.9 g/g of rat liver (31, 32). The available evidence suggests that the hydroxylase is...
rate could be elicited by phenylalanine (Fig. 4A, □ and ▲). This may be related to the fact that physiological concentrations of BH₄ are subsaturating. According to the data of Fig. 4B, 3 μM and 20 μM BH₄ saturate 60 and 90%, respectively, of the BH₄-binding sites in the absence of phenylalanine versus 25 and 70% at a high concentration of phenylalanine. This means that, due to the unfavorable energy of coupling between phenylalanine and BH₄, just described, phenylalanine, in addition to its own direct positive effect on the phosphorylatability of the hydroxylase, can act indirectly by decreasing the binding of the negative effector BH₄.

It was finally noted that hydroxylase which had been preincubated with phenylalanine and then exposed to BH₄, was a better substrate than enzyme that had been preincubated with BH₄ and then exposed to phenylalanine (Fig. 4B; see also Fig. 7).

The Effect of Ligands on the Reversal of the Kinase Reaction—The reversal of the protein kinase reaction (28, 29) was studied by incubation of the 32P-labeled phosphohydroxylase with a high concentration of MgADP. In order to avoid interference from the forward reaction, the concentration of hydroxylase was kept low (0.1 μM). Furthermore, unlabeled ATP (20 μM) was included to isotopically dilute the 32P ATP formed in the reaction. Whether the ligands BH₄ and phenylalanine were present or not, more than 95% of the 32P of the phosphoenzyme could be removed in a kinase/ADP-dependent manner (Fig. 5, and data not shown).

The presence of BH₄ lowered the rate of dephosphorylation of phosphorylated enzyme (Fig. 5) to 0.5–0.7 times (range of three experiments) the rate observed in the absence of added ligand. Phenylalanine increased the rate of dephosphorylation by a factor of 1.2–1.3 (range of three experiments).

Activation of Phenylalanine 4-Monoxygenase in the Absence and Presence of BH₄—Comparison of the Phosphorylated and Nonphosphorylated Form of the Enzyme—The data located in the cytosol matrix of hepatocytes. The subcellular distribution of BH₄ is not known, but it seems reasonable to assume a distribution in the cytosol matrix and nuclear matrix. Morphometric analysis (33) indicates that 45% of the liver volume is occupied by hepatocyte cytosol matrix and 53% by hepatocyte cytosol and nuclear matrix. A rough approximation of the concentration of the hydroxylase and BH₄, in the relevant compartments would then be 1.1 mg/liter and 5.4 μg/liter, respectively. Taking the Mr of the subunit of the hydroxylase to be 51,000 (1, 15) and that of BH₄ to be 241, the concentration of the hydroxylase should be 21.8 μM and that of BH₄ 22.4 μM. If the hydroxylase is considered to be the main intracellular binder of BH₄ in hepatocytes and to have one binding site for the cofactor per subunit, the concentration of unbound BH₄ could theoretically vary between 0.6 and 22.4 μM depending on the degree of saturation of the hydroxylase with respect to cofactor.

![Fig. 4. The rate of phosphorylation of phenylalanine 4-monoxygenase as a function of the concentration of phenylalanine. The rate of phosphorylation of the hydroxylase (1–2 μM) by C subunit (2–10 nM) was determined at various concentrations of phenylalanine and no BH₄ (□), 5 μM (▲), 20 μM (●), or 130 μM (△) BH₄. A, the incubations were started by adding a mixture of hydroxylase and kinase. B, 130 μM BH₄ was present during the assay, which was started by the addition of hydroxylase that had been preincubated for 10 min at 30 °C in Hepes buffer with either 130 μM BH₄ (△) or with various concentrations of phenylalanine (●). In the latter case (●), the composition of the reaction mixture was adjusted so that the concentration of phenylalanine was the same during preincubation and during the ensuing phosphorylation reaction. C, Hill plots of the data presented in the left-hand curve of A (□) and the two curves (△, ▲) of B. Y = Vₒ × Vₒ/Vₘₐₓ = Vₒ, which is the fractional increase of phosphorylation rate seen at the concentration x of phenylalanine. Vₒ is the rate in the absence of phenylalanine and Vₘₐₓ the rate in the presence of a presumed saturating concentration of phenylalanine under a particular set of conditions.

![Fig. 5. The reversal of the protein kinase reaction. 32P-labeled phosphohydroxylase (0.1 μM), prepared as described under "Experimental Procedures," was incubated at 30 °C in Hepes buffer containing 10 mM Mg acetate, 5 mM ADP, 20 μM ATP, and 0.5 μM C subunit. The incubations contained either no effector (●), 100 μM BH₄ (△), or 1 mM phenylalanine (▲). Either the kinase (△) or ADP (●) was omitted from the control incubations. The rate of dephosphorylation of the 32P-labeled hydroxylase was measured by spotting aliquots (4μl) on phosphocellulose strips (21). Similar results were obtained when the degree of dephosphorylation was assayed by the amount of 32P becoming soluble in 10% trichloroacetic acid.]
The apparent Hill coefficient with an optimal concentration of phenylalanine. The apparent Hill coefficient with an optimal concentration of phenylalanine, and the enzyme preincubated with the concentration for phosphorylated hydroxylase activity as described under "Experimental Procedures," or nonphosphorylated enzyme, was present (Fig. 6).

**FIG. 6. The potency of phenylalanine as an activator of the phosphorylated and nonphosphorylated form of phenylalanine-4-monooxygenase.** A, phosphorylase (solid symbols), prepared as described under "Experimental Procedures," or nonphosphorylated enzyme (open symbols), both present at a concentration of 2 μM, were preincubated for 5 min at 25 °C with various concentrations of phenylalanine (abscissa). The preincubations contained no BH₄ (O, ●) or 4 μM BH₄ (C, △). Aliquots were tested for their hydroxylase activity as described under "Experimental Procedures." A, the activity of phosphorylated and nonphosphorylated enzyme as a function of preincubation conditions. The activity of enzyme preincubated in the absence of BH₄ and phenylalanine was taken as unity. Half-maximal activation of the phosphorylated and nonphosphorylated forms of the hydroxylase required 29 and 51 μM phenylalanine, respectively. These values were the same whether the preincubation time was 5 or 10 min. When BH₄ was present, the corresponding values were 66 and 93 μM for 5 min of preincubation and slightly higher for 10 min of preincubation. B, a Hill plot of the data obtained for phosphorylated (●) and nonphosphorylated (O) enzyme preincubated in the absence of BH₄. Y = the fractional activation of the hydroxylase = ω/Vₘₐₓ - ω₀, where ω represents the activity of the enzyme preincubated with the concentration ω₀ of phenylalanine, ω₀ represents the activity of the enzyme preincubated in the absence of phenylalanine, and Vₘₐₓ represents the activity after preincubation with an optimal concentration of phenylalanine. The apparent Hill coefficient was 3 for the phosphorylated enzyme and 2.6 for the nonphosphorylated enzyme.

shown (Fig. 6, O and △) for the activation of the hydroxylase activity of nonphosphorylated enzyme by phenylalanine were obtained under conditions similar to those used to test the stimulation by phenylalanine of the phosphorylation of the hydroxylase (Fig. 4A, O and △).

Comparison of the two sets of data (Table II) show a very similar dependence on the phenylalanine concentration for the two processes. Furthermore, the effect of phenylalanine revealed an apparent positive cooperativity in either case (Fig. 4C and Fig. 6B).

The recent observation (12, 13) that the phosphorylated form of the hydroxylase required less phenylalanine to be activated was confirmed and shown to be true also when the physiological cofactor BH₄ was present (Fig. 6).

**DISCUSSION**

A large number of intracellular proteins are subject to phosphorylation by the catalytic subunit of cAMP-dependent protein kinases (34, 35). One way in which activation of this single type of kinase subunit may lead to various patterns of phosphorylation, depending on the metabolic state of the cell, is by metabolites interacting with substrate proteins whose susceptibility towards phosphorylation is thereby altered. The present study shows phenylalanine 4-monooxygenase to be under such substrate-directed control of phosphorylation, which has so far been described only for a few enzymes (36, 37), none of which are involved in amino acid metabolism.

Incorporation of phenylalanine 4-monooxygenase with L-phenylalanine made the enzyme more readily phosphorylated by the C subunit of cAMP-dependent protein kinase (Figs. 1 and 2). Such modulation of the phosphorylation of the hydroxylase may also occur in vivo, since phenylalanine acted at physiologically relevant (38) concentrations and was efficient in the presence of the natural cofactor BH₄ (Fig. 4). The hydroxylase is considered to have three types of sites capable of interacting with phenylalanine: a regulatory site responsible for the activation of the enzyme by phenylalanine (5, 6), the catalytic site, and a low-affinity site presumed to mediate enzyme inhibition by millimolar concentrations of phenylalanine (39). The strikingly similar dependence on phenylalanine concentration (Table II) for the activation of the hydroxylase (Fig. 6) and the modulation of its rate of phosphorylation (Fig. 4), suggested that the same site, i.e. the regulatory site, mediated both effects of phenylalanine. This conclusion is further supported by the positive cooperativity shown by phenylalanine for both effects (Figs. 4, 6). Another clue as to whether the regulatory or the catalytic site was responsible for the enhanced phosphorylation came from the observation that in complex with the nonphosphorylated hydroxylase, the phosphoenzyme required less phenylalanine to be activated (Fig. 6, Table II), although it revealed the same Kₘ value for phenylalanine in the hydroxylation reaction (7; and data not shown). Based on simple considerations of thermodynamic reciprocity (27) it follows that the binding of phenylalanine to the regulatory site, but not to the catalytic site, should shift the equilibrium between nonphosphorylated and phosphorylated enzyme in favor of the latter. Such a shift occurs if phenylalanine preferentially enhances the forward reaction of Equation 3.

\[
\text{Hydroxylase + Mg[y-32P]ATP} \rightleftharpoons \text{32P-hydroxylase + MgADP} \tag{3}
\]

In fact, phenylalanine stimulated the forward reaction by a factor of 2 (Figs. 1, 2, and 4) as compared to 1.2-1.3 for the reverse reaction (Fig. 5). It follows that phenylalanine favored the phosphorylated form of the hydroxylase, in accordance with its action being exerted through the regulatory site. A preliminary "mapping" of this site (Table I) showed that it had a considerably (at least 500 times) higher affinity for the L-isomer than the D-isomer of phenylalanine. Whereas L-2-thienylalanine had a higher affinity than p-chlorophenylalanine for the regulatory site (Table I), the converse is true for the catalytic site (39). This means that the two sites differ considerably in the structure facing the phenyl moiety of phenylalanine. There has been some controversy as to whether tryptophan is an activator of the hydroxylase (5, 6). The data of Table I support the contention (6) that this amino acid (at supraphysiological concentrations) does bind to the regulatory site.

Physiologically relevant concentrations of the natural cofactor BH₄ decreased the rate of the cAMP-dependent phosphorylation (Figs. 1 and 4) as well as the dephosphorylation
The concentrations of effector required for half-maximal modulation of the state of activation of the phosphorylatability of phenylalanine 4-monoxygenase

The upper row gives the concentrations of phenylalanine required for half-maximal activation of the hydroxylase activity of the nonphosphorylated and phosphorylated (values in parentheses) form of the enzyme. The values refer to enzyme preincubated in the absence of BH₄ or presence of 4 μM BH₄ and are calculated from Hill plots of the curves shown in Fig. 6A. The middle row gives the concentrations of phenylalanine half-maximally enhancing the rate of phosphorylation of the hydroxylase at various concentrations 0, 3, and 130 μM of BH₄. The values were calculated from the plots of Figs. 4, A and B. The lower row gives the concentrations of BH₄, half-maximally decreasing the rate of phosphorylation of the hydroxylase, as calculated from the experiments shown in Fig. 3.

| Parameter studied          | Concentration of effector required for half-maximal modulation | Symbol used | Other effectors present |
|----------------------------|---------------------------------------------------------------|-------------|-------------------------|
| Activation of the hydroxylase | 51 μM Phe (29 μM)³ | Kₑ (Phe)   | 4 μM BH₄                |
| Increase in rate of phosphorylation of the hydroxylase | 93 μM Phe (66 μM)³ | Kₑ (Phe)   | 3 μM BH₄                 |
| Decrease in rate of phosphorylation of the hydroxylase | 180 μM Phe | Kₑ (Phe/BH₄) | 130 μM BH₄              |
|                            | 2.3 μM BH₄ | Kₑ (BH₄)   | 10 μM Phe                |
|                            | 8.3 μM    | Kₑ (BH₄/Phe)|                        |

*The values in parentheses refer to the phosphorylated form of the hydroxylase.

Preincubation of the hydroxylase with a high concentration of BH₄ virtually blocks the activity effect (4, 6, 25), but not the phosphorylation-modulating effect (Fig. 4B) of phenylalanine. This means that the BH₄-saturated hydroxylase remains in a low activity state (termed BP in Fig. 7) even when phenylalanine is bound to the regulatory site. Using the rate of phosphorylation of the hydroxylase as a probe, this doubly liganded state differs from the doubly liganded phenylalanine-activated state (PB) as well as from the mono-liganded P- and B-states (see Fig. 7 for details). The latter (P, PB, and B) all differ from the ground state (G) in their phosphorylatability (Fig. 7). Previous studies have provided ample evidence that the conformation of the P-state differs from that of the G-state (4, 10, 40, 42). A recent study using the susceptibility to chymotrypsinolysis as a conformational probe also concluded that the B-state of the hydroxylase differed from the G-state, but found no difference between states P and PB (42). This suggests that the binding of BH₄ to the native hydroxylase leads to a state (B) in which both the site(s) of chymotrypsin cleavage and the site of phosphorylation have an altered microenvironment. When BH₄ binds to the phenylalanine-activated enzyme, the putative conformational change (from P to PB) may be more subtle, i.e. the change may be limited to the site of phosphorylation only. An alternative explanation, i.e. that BH₄ interferes sterically with the phosphorylation, is unlikely because the synthetic cofactor 6,7-dimethyltetrahydropterin, which is a slightly bulkier molecule than BH₄ and binds to the same site, does not inhibit the phosphorylation reaction (Table 1).

Fig. 7. Schematic representation of the phosphorylatability of phenylalanine 4-monoxygenase in various states of ligation. The ordinate indicates the relative rate of phosphorylation of the hydroxylase (data extracted from Figs. 3 and 4) at various ligational and conformational states of the enzyme. In the absence of effectors the enzyme is considered to be in the ground state, G. Binding of phenylalanine to the regulatory site of the hydroxylase under activating conditions (4) brings the enzyme to the P-state. Binding of BH₄ to enzyme in the P-state leads to the doubly liganded PB-state. If the enzyme is first exposed to BH₄ (B-state) and then to phenylalanine, a doubly liganded state (BP) occurs which differs from the PB-state in its phosphorylatability. For the sake of simplicity, only one subunit of the oligomeric enzyme is considered in the model.

The magnitude of the phosphorylation enhancement by phenylalanine was 2–3-fold depending on the concentration of BH₄ present (Fig. 4). This may seem a modest effect, but as pointed out by Goldbeter and Koshland (43), there is an added sensitivity inherent in covalent modification schemes when one of the converter enzymes operates in the "zero-order" region, i.e. the region of saturation with respect to protein substrate. Phosphohydroxylase is dephosphorylated by a phosphatase (11), and the activity of this phosphatase may approach that predicted from zero-order (43) kinetics. Thus, the phenylalanine hydroxylase of rat liver hepatocytes, exposed to a brief stimulation of the cAMP-dependent protein kinase, remains in a phosphorylated condition after the activity of the cAMP-dependent kinase has returned to its basal level (44).

In conclusion, the data presented in this study indicate that phenylalanine 4-monoxygenase is subject to an interwoven control by allosteric binding of ligands and covalent modification by phosphorylation. Thus, an increase of the phenyl-

Table II

By the concentrations of effector required for half-maximal modulation of the state of activation of the phosphorylatability of phenylalanine 4-monoxygenase.

| Parameter studied          | Concentration of effector required for half-maximal modulation | Symbol used | Other effectors present |
|----------------------------|---------------------------------------------------------------|-------------|-------------------------|
| Activation of the hydroxylase | 51 μM Phe (29 μM)³ | Kₑ (Phe)   | 4 μM BH₄                |
| Increase in rate of phosphorylation of the hydroxylase | 93 μM Phe (66 μM)³ | Kₑ (Phe)   | 3 μM BH₄                 |
| Decrease in rate of phosphorylation of the hydroxylase | 180 μM Phe | Kₑ (Phe/BH₄) | 130 μM BH₄              |
|                            | 2.3 μM BH₄ | Kₑ (BH₄)   | 10 μM Phe                |
|                            | 8.3 μM    | Kₑ (BH₄/Phe)|                        |
Modulation of the Phosphorylation of Phenylalanine 4-Monoxygenase

Alanine concentration not only activates the enzyme (3, 4, Fig. 6), but also enhances its phosphorylation (Fig. 4). The phosphorylated enzyme is in turn sensitized towards the effect of amino acid analogs) in his laboratory, and to Martha Bass and Dr. T. Soderling for help in the determination of chemical phosphate.

Acknowledgments—We are indebted to Dr. J. D. Corbin who generously allowed us to carry out part of this work (dealing with the effect of amino acid analogs) in his laboratory, and to Martha Bass and Dr. T. Soderling for help in the determination of chemical phosphate.

REFERENCES
1. Kaufman, S. (1971) Adv. Enzymol. 35, 245–319
2. Hasegawa, H., Imaizumi, S., Ichiyama, A., Sugimoto, T., Matsura, S., Oka, K., Kato, T., Nagatsu, T., and Akino, M. (1979) in Chemistry and Biology of Pteridines (Kisliuk, R., and Pfeiderer, W., eds) pp. 183–188, Elsevier/North-Holland, New York
3. Nielsen, K. H. (1969) Eur. J. Biochem. 7, 360–369
4. Shimna, R., and Gray, D. W. (1980) J. Biol. Chem. 255, 4793–4800
5. Shimna, R. (1980) J. Biol. Chem. 255, 10029–10032
6. Kaufman, S. and Mason, K. (1982) J. Biol. Chem. 257, 14667–14678
7. Abita, J.-P., Milstien, S., Chang, N. and Kaufman, S. (1976) J. Biol. Chem. 251, 5310–5314
8. Øgren, D., Dæskeland, S. O., and Miller, J. P. (1983) J. Biol. Chem. 258, 1041–1049
9. Donlon, J. and Kaufman, S. (1978) J. Biol. Chem. 253, 6657–6659
10. Garrison, J. C. and Wagner, J. D. (1982) J. Biol. Chem. 257, 13135–13143
11. Kaufman, S., Hasegawa, H., Wilgus, H., and Paraniak, M. (1981) Cold Spring Harbor Conf. Cell Proliferation 8, 1391–1406
12. Shimna, R., Mortimore, G. E., Schwerer, C. M., and Gray, D. W. (1982) J. Biol. Chem. 257, 11213–11216.
13. Dæskeland, A., Dæskeland, S. O., Øgren, D., and Flattmark, T. (1983) Fed. Proc. 42, 2193.
14. Bailey, S. W. and Aylng, J. E. (1978) J. Biol. Chem. 253, 1596–1605
15. Dæskeland, A., Ljones, T., Skotland, T. and Flattmark, T. (1982) Neurochem. Res. 7, 407–421
16. Shimna, R., Gray, D. W. and Pater, A. (1979) J. Biol. Chem. 254, 11306–11306
17. Stull, J. T. and Buss, J. E. (1977) J. Biol. Chem. 252, 851–857
18. Øgren, D. and Dæskeland, S. O. (1981) FEBS Lett. 129, 282–286
19. Corbin, J. D. and Reimann, E. M. (1974) Methods Enzymol. 38, 287–290
20. Fossberg, T. M., Dæskeland, S. O., and Ueland, P. M. (1978) Arch. Biochem. Biophys. 189, 372–381
21. Roskoski, R. (1983) Methods Enzymol. 99, 3–6
22. Bailey, S. W., and Aylng, J. E. (1980) Anai. Biochem. 107, 156–164
23. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
24. Kaufman, S., and Lovenberg, B. (1959) J. Biol. Chem. 234, 2683–2688
25. Aylng, J. E., and Helland, G. D. (1975) in Chemistry and Biology of Pteridines (Pfeiderer, W., ed) pp. 305–319, Walter de Gruyter, Berlin
26. Wyman, J. (1965) J. M. Biol. 11, 631–644
27. Weber, G. (1975) Adv. Protein Chem. 29, 1–83
28. Rabinowitz, M., and Lipmann, F. (1960) J. Biol. Chem. 235, 1043–1050
29. Shizuta, Y., Beavo, J. A., Bechtel, P. J., Hofmann, F. and Krebs, E. G. (1976) J. Biol. Chem. 250, 6891–6896
30. Milstien, S., and Kaufman, S. (1975) J. Biol. Chem. 250, 4777–4781
31. Kaufman, S., Holtzman, N. A., Milstien, S., Butler, I. J., and Krumholz, A. (1975) N. Engl. J. Med. 283, 785–790
32. Brautigam, M., and Dreessen, R. (1982) Hoppe- Seyler's Z. Physiol. Chem. 363, 1200–1207
33. Blouin, A., Bolender, R. P., and Weibel, E. R. (1977) J. Cell Biol. 72, 441–445
34. Krebs, E. G. and Beavo, J. A. (1979) Annu. Rev. Biochem. 48, 923–959
35. Cohen, P. (1982) Nature (Lond.) 296, 613–620
36. Berglund, L., Ljungström, O., and Engström, L. (1977) J. Biol. Chem. 252, 613–619
37. El-Maghribi, M. R., Claus, T. H., and Pilkis, S. J. (1983) Methods Enzymol. 99, 212–219
38. Jefferson, L. S., Robertson, J. W., and Tolman, E. L. (1971) Excerpta Med. Int. Congr. Ser. 244, 106–123
39. Dhondt, J. L., Dautrevaux, M., Biserte, G., and Farriaux, J. P. (1978) Biochimie 60, 787–794
40. Fisher, D. B. and Kaufman, S. (1979) J. Biol. Chem. 248, 4345–4353
41. Paraniak, M. A., and Kaufman, S. (1981) J. Biol. Chem. 256, 6876–6882
42. Phillips, R. S., Iwaki, M., and Kaufman, S. (1983) Biochem. Biophys. Res. Commun. 110, 919–925
43. Goldbeter, A. and Kosland, D. E., Jr. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 6840–6844
44. Reed, B. Y., Wilgus, H., Phillips, R. and Kaufman, S. (1983) Fed. Proc. 42, 2029