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# Further studies of the role of Ser-16 in the regulation of the activity of phenylalanine hydroxylase

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**ABSTRACT** **It was previously proposed that the activation of rat liver phenylalanine hydroxylase (EC 1.14.16.1) by cAMP-dependent protein kinase-mediated phosphorylation of Ser-16 is due to the introduction of the negatively charged phosphate group. To explore the validity of this proposal, we have applied site-directed mutagenesis to specifically replace Ser-16 with negatively charged amino acids, glutamic and aspartic; with polar uncharged amino acids, asparagine and glutamine; with the positively charged amino acid lysine; and with the nonpolar hydrophobic amino acid alanine. The wild-type and mutant enzymes were purified to homogeneity, and the importance of Ser-16 in the activation of phenylalanine hydroxylase was examined by comparing the state of activation of the phosphorylated form of the wild-type hydroxylase with that of the mutants. The kinetic studies carried out on the wild-type phosphorylated hydroxylase showed that all the activation could be accounted for by an increase in $V_{max}$ with no change in $K_m$ for either phenylalanine or the pterin cofactor. Replacement of Ser-16 with a negatively charged residue, glutamate of aspartate, resulted in the activation of the hydroxylase by 2- to 4-fold, whereas replacement with glutamine, asparagine, lysine, or alanine resulted in a much more modest increase. Further, lysolecithin was found to stimulate the phosphorylated hydroxylase and the mutant enzymes S16E and S16D by a factor of 6–7. In contrast, the mutants S16Q, S16N, and S16A all showed the same magnitude of activation as the wild-type with lysolecithin. Therefore, this study demonstrates that activation of the enzyme by phosphorylation of Ser-16 by cAMP-dependent protein kinase is due to the introduction of negative charge(s) and strongly suggests the involvement of electrostatic interaction between the regulatory and catalytic domains of the hydroxylase.**

Phenylalanine hydroxylase [PAH; phenylalanine 4-monoxygenase; L-phenylalanine, tetrahydrobiopterin:oxygen oxidoreductase (4-hydroxylating), EC 1.14.16.1] catalyzes the conversion of phenylalanine to tyrosine (1). The absence of PAH results in phenylketonuria, a disease that is characterized by marked elevation of phenylalanine levels that, if untreated, can cause severe mental retardation (2, 3). Phosphorylation of PAH mediated by cAMP-dependent protein kinase (PKA) has been shown to increase $V_{max}$ by a factor of 3–4. The site of phosphorylation has been identified as Ser-16, located in the N-terminal region of the enzyme (4). Phosphorylation-mediated activation of hepatic PAH has also been demonstrated *in vivo* in rats treated with glucagon (5). In addition, PAH can be activated by incubation with certain phospholipids such as lysolecithin (6) and by interaction with phenylalanine (7–10). The enzyme can be deactivated by its natural pterin cofactor, (6*R*)-5,6,7,8-tetrahydrobiopterin ($BH_4$) (11). The consequences of activation of PAH by phosphorylation, phenylalanine, and limited proteolysis have been reviewed by Kaufman (1).

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Recently, it has been reported that recombinant rat liver PAH is activated about 4-fold by the replacement of Ser-16 with a negatively charged glutamate residue. It was suggested that electrostatic interactions might be involved in the activation of the enzyme by phosphorylation (12). In the present study, we have investigated in greater detail the nature of activation of recombinant rat liver PAH resulting from the phosphorylation of Ser-16 mediated by PKA. To determine whether this type of activation results from the introduction of negative charges into this region of the hydroxylase molecule, we have by site-directed mutagenesis replaced Ser-16 with a negatively charged (glutamate or aspartate), uncharged (glutamine, asparagine, or alanine), or positively charged (lysine) amino acid. The state of activation of these mutant enzymes and their ability to be further activated by incubation with lysolecithin have been studied. The results strongly support the conclusion that phosphorylation-mediated activation of PAH is due primarily to the negative charges introduced by the phosphate group. These studies also indicate that the size of the amino acid residue at position 16 can affect the state of activation of the hydroxylase.

## MATERIALS AND METHODS

**Materials.** pLNC209 is a bacterial plasmid clone that expresses rat liver PAH (ref. 13 and Fig. 1). Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs, Bethesda Research Laboratories, Promega, and United States Biochemical. The *Escherichia coli* host strain was DH5 [$F^-$ *endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ*(lacZY-argF)U169*] (GIBCO/BRL). Phenylalanine, lysolecithin, D-glucose 6-phosphate, D-glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* dihydropteridine reductase and PKA catalytic subunit were obtained from Sigma. $BH_4$ and 6-methyl-5,6,7,8-tetrahydropterin ($6MPH_4$) were obtained from B. Shirck's laboratory (Jona, Switzerland). Catalase was purchased from Boehringer Mannheim.

**Oligonucleotide-Directed Site-Specific Mutagenesis.** A segment encoding the N-terminal sequence of rat live PAH was excised with *Eco*RI and *Xho* I restriction enzymes and subsequently ligated to *Eco*RI/*Xho* I-digested replicative form of pCRII vector (Novagen) to generate a template for mutagenesis. A phagemid *in vitro* mutagenesis kit (Bio-Rad) was used as recommended by the manufacturer. The sequences of the mutagenic primers were as follows: 0343 [$Ser^{16} \rightarrow$ Asp (S16D)], 5′-CCCAAAGTCCTGGAGTTTTCTG-3′; 0344 (S16N), 5′-CCCAAAGTCATCGAGTTTTCTG-3′; 0345 (S16Q), 5′-CCCAAAGTCGTTGAGTTTTCTG-3′; 0355 (S16A), 5′-CCCAAAGTCCGCGAGTTTTCTG-3′; 0356 (S16K), 5′-CCCAAAGTCTTTGAGTTTTCTG-3′. Single-stranded uracil-labeled templates were prepared as described (14).

Abbreviations: PAH, phenylalanine hydroxylase; PKA, cAMP-dependent protein kinase; $BH_4$, (6*R*)-5,6,7,8-tetrahydrobiopterin; $6MPH_4$, 6-methyl-5,6,7,8-tetrahydropterin.

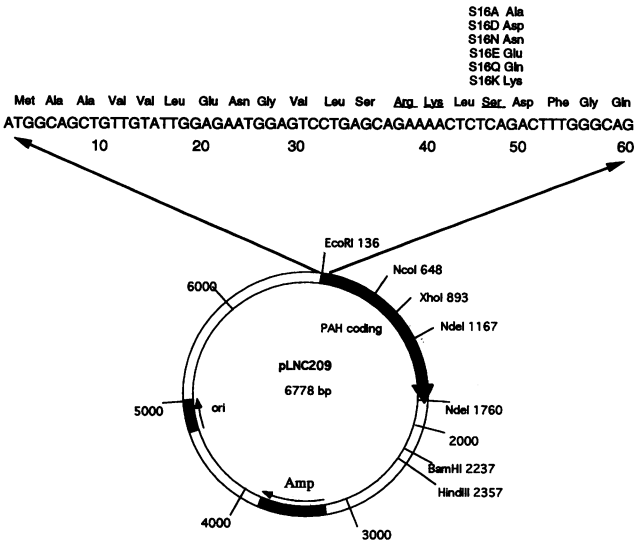


FIG. 1. Site-directed mutagenesis of the rat PAH gene in the expression vector pLNC209. The protein sequence spans the putative phosphorylation site; the consensus sequence for the PKA phosphorylation site is underlined (4).

**DNA Sequencing.** All mutants were screened by dideoxy sequencing (15) of double-stranded DNA prepared by Magic Minipreps (Promega). The products were analyzed on an Acugen automated DNA sequencer (EG&G, Salem, MA).

**Growth of Bacteria.** *E. coli* DH5 transformed with each of the mutant plasmids generated was grown aerobically at 37°C in Luria–Bertani (LB) medium [0.5% NaCl/1% tryptone (Difco)/0.5% yeast extract (Difco)] containing ampicillin (100 μg/ml) and ferrous ammonium sulfate (0.1 mM). For large-scale production of the recombinant proteins, cells were grown in 300 liters of LB/ampicillin/iron medium at 37°C for 6 hr after addition of 1% inoculum and 0.5 mM isopropyl β-D-thiogalactopyranoside. The cells were harvested at 0°C by centrifugation and washed with 10 mM $MgSO_4$.

**Enzyme Purification.** The wild-type and mutant hydroxylases were purified essentially as described (16, 17). Thirty-five grams of frozen *E. coli* cells were suspended in 100 ml of homogenization buffer (0.03 M Tris·HCl, pH 7.6/0.15 M KCl/1 mM EDTA/0.2 mM phenylmethylsulfonyl fluoride/35 mg of lysozyme/6 μg of bovine pancreas deoxyribonuclease I/50 μg of leupeptin/70 μg of pepstatin/50 μg of aprotinin/0.02 mM phenylalanine). The mixture was then sonicated in ice for 10 min in 30-sec on/off cycles with an Ultrasonics (Farmingdale, NY) model W-225 processor and a C2 probe (Heat Systems/Ultrasonics). After sonication, the homogenate was centrifuged for 30 min at 25,000 × *g* in an SS-34 rotor in a Sorvall RC-5B centrifuge. The pellet was discarded. The supernatant was loaded on a phenyl-Sepharose affinity column and then washed and eluted as described (17). The fractions with the highest activity were pooled and applied to a DEAE-cellulose column (2.5 cm × 10 cm) and then eluted by a gradient of 0–0.4 M KCl in 0.03 M Tris·HCl (pH 7.6). The enzyme was concentrated by ultrafiltration. Protein was quantitated with the Pierce Bradford assay with bovine serum albumin as standard. Purified enzymes were subjected to SDS/10% polyacrylamide gel electrophoresis (18) (see Fig. 2*A*).

**Immunological Analysis.** Samples (2 μg) of purified wild-type and mutant proteins were subjected to SDS/polyacrylamide gel electrophoresis and subsequently transferred to a nitrocellulose membrane. After transfer, the membrane was preincubated at room temperature for 1 hr in 20 ml of Tris-buffered saline (TBS: 0.1 M Tris·HCl, pH 7.5/0.9% NaCl) containing 0.1% Tween 20. The membrane was then washed three times for 10 min with TBS at room temperature and incubated with sheep anti-rat PAH antibody from Pierce (1:250). The membrane was then washed as described above and incubated with horseradish peroxidase-conjugated rabbit anti-sheep IgG (1:200). Finally, the membrane was washed with 20 ml of TBS three times over a 15-min span, and the antibody complexes were visualized with $H_2O_2$ and 4-chloro-1-naphthol (see Fig. 2*B*).

**Enzyme Assays.** Initial rates for the phenylalanine-dependent oxidation of NADH were determined spectrophotometrically at 340 nm with a Gilford 250 spectrophotometer that was temperature controlled by a Neslab Instruments (Portsmouth, NH) RTE-110 waterbath (16). Enzyme assays were performed in 0.1 M potassium phosphate buffer (pH 6.8) at 25°C at atmospheric oxygen tension. The assay mixture contained 0.01% catalase, 0.125 mM NADH, and an excess of sheep liver dihydropteridine reductase. The range of L-phenylalanine concentrations was from 0.02 to 1 mM in the presence of 0.02 mM $BH_4$, and the range of $BH_4$ was from 0.001 to 0.01 mM in the presence of 1 mM L-phenylalanine. An extinction coefficient of 6220 $M^{-1}\cdot cm^{-1}$ was used to calculate the amount of NADH oxidized. The initial rates were corrected for the contribution due to any autoxidation of $BH_4$. The specific activities of the wild-type and the mutant enzymes were measured by the colorimetric determination of tyrosine formation (19). The enzymes were assayed at 25°C for 30 min in the presence of 6MP$H_4$ as cofactor. The reaction mixture (1 ml) contained 0.1 M phosphate buffer (pH 6.8), 5 mM L-phenylalanine 0.01% catalase, 0.35 mM NADH, 10 mM glucose 6-phosphate, and excess dihydropteridine reductase and glucose-6-phosphate dehydrogenase. The reactions were stopped by the addition of 12% trichloroacetic acid and the tyrosine formed was measured by the nitrosonaphthol method (20).

**Phosphorylation.** Recombinant rat liver PAH was phosphorylated at 30°C in a reaction mixture (0.2 ml) containing 10 mM Tris·HCl (pH 7.4), 1 mM $MgCl_2$, 0.8 mM ATP, 10 μg of PKA catalytic subunit, and 200 μg of the hydroxylase. Aliquots (10 μl) were withdrawn from the reaction mixture at 10-min intervals to measure activity as described above.

**Analysis of Kinetic Data.** Kinetic parameters were computed by direct fitting of experimental data to the Michaelis–Menten equation with ULTRAFIT version 2.1 (Biosoft, Cambridge, U.K.), a nonlinear curve-fitting program with statistical analysis for the Apple Macintosh computer.

## RESULTS

**Construction and Verification of Mutants.** We used site-directed mutagenesis to convert the phosphorylation site, Ser-16, in the regulatory domain of recombinant rat liver PAH to alanine, glutamic acid, aspartic acid, asparagine, glutamine, or lysine. For this purpose, pLNC209, a recombinant plasmid that expresses wild-type PAH was double-digested with *Eco*RI and *Xho* I restriction endonucleases. pLNC209 (6778 bp) has an *Eco*RI site at position 136 and an *Xho* I site at 893. The *Eco*RI–*Xho* I DNA fragment was purified and ligated to pCRII (Novagen) which had been cut with *Eco*RI and *Xho* I to generate single-stranded uracil-labeled template for mutagenesis. After propagation in *E. coli* DH5, recombinant plasmid DNA was isolated, and the mutations were verified by sequencing the subcloned double-stranded DNA. The mutated *Eco*RI–*Xho* I fragments were finally subcloned into the *Eco*RI–*Xho* I site of the expression vector pLNC209. Mutant S16E was constructed and verified as described (12).

**Expression of PAH Mutants.** The wild-type parent plasmid and each mutant plasmid were then expressed in large-scale cultures and the expressed enzymes were isolated and purified to homogeneity (13). Western blot analysis of the hydroxylase prepared from *E. coli* DH5 expressing individual mutants demonstrated that all the mutant proteins were present at

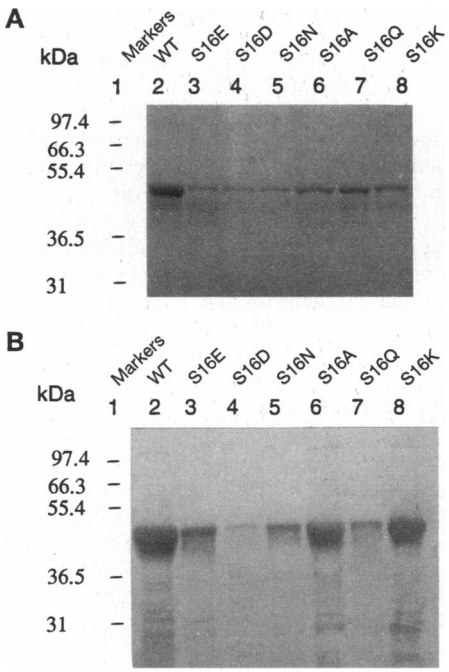


FIG. 2. Electrophoretic behavior of purified wild-type PAH (WT) and the substitution mutants. Coomassie blue-stained SDS/10% polyacrylamide gel (*A*) and the corresponding Western blot probed with sheep antibody to rat PAH (*B*) are shown.

levels comparable to the wild type (Fig. 2). The specific activities of the wild type and the mutants measured in the presence of $6MPH_4$ ranged from 4.9 to 5.2 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ (Table 1).

**Activation of Recombinant Rat Liver PAH by Phosphorylation.** Activation of PAH was observed only when assayed in the presence of $BH_4$. When measured on aliquots after various times of incubation under phosphorylation conditions, the hydroxylase activity reached a maximum increase of 3.3-fold after 30 min (data not shown). Further, the rate of activation was found to depend on the amount of the catalytic subunit of PKA but the maximum activation reached was independent of the amount of kinase used. The apparent $K_m$ of the phosphorylated enzyme for $BH_4$ was 1.6-fold higher than that of the wild-type hydroxylase. This result differs from that previously reported (21), where no change in $K_m$ after phosphorylation was detected for PAH isolated from rat liver. This difference may reflect the fact that the native enzyme is already partially phosphorylated (21). In agreement with the results obtained with the enzyme from liver (21), no change in the apparent $K_m$ for phenylalanine was found when it was measured at a saturating concentration of $BH_4$ (Table 2).

**Kinetic Studies of the Mutant and Wild-Type Enzymes.** To evaluate the mechanism responsible for the increase in catalytic activity in the phosphorylated hydroxylase, we determined the kinetic parameters of each of the mutants and the wild type. These kinetic parameters were calculated from initial rates measured at pH 6.8 for a constant concentration of the cofactor $BH_4$ and various concentrations of L-phenylalanine or for a constant concentration of L-phenylalanine and various concentrations of $BH_4$. There was a slight variation in the apparent $K_m$ for L-phenylalanine between the wild-type enzyme and the mutants (Table 2). The apparent $K_m$ values for L-phenylalanine of the mutants and the wild type in the presence of the cofactor $BH_4$ are in the range 180–290 $\mu M$. The apparent $K_m$ values for $BH_4$ of the mutants in the presence of L-phenylalanine are in the range 2.5–4.6 $\mu M$. Replacement of Ser-16 with a singly negatively charged residue, glutamate or aspartate, resulted in a severalfold activation in enzymatic activity with respect to the activity of the wild-type enzyme. Most of these effects can be accounted for by an increase in $V_{max}$. The phosphorylated enzyme showed a 3.3-fold increase in $V_{max}$. This value is about the same as that observed following the phosphorylation-mediated activation of the wild-type recombinant enzyme (13). In addition to the relatively large activation resulting from the replacement of Ser-16 with a negatively charged residue, Table 2 shows that a modest degree of activation resulted from replacement of Ser-16 with an uncharged residue such as glutamine or asparagine or the positively charged lysine. Substitution of alanine for Ser-16, however, did not result in activation. As shown in Fig. 3, the extent of activation increased with the molecular volume of these uncharged or positively charged residues, as reflected by their average area (22). The activating effect due to the increased

Table 1. Specific activities of wild-type and mutant forms of recombinant rat liver PAH obtained by transfection of *E. coli* DH5 with pLNC209

| Enzyme | Specific activity, $\mu mol \cdot min^{-1} \cdot mg^{-1}$ |
|---|---|
| Wild type | 4.9 |
| S16E | 5.2 |
| S16Q | 5.2 |
| S16S(*P*) | 5.0 |
| S16N | 5.0 |
| S16D | 5.0 |
| S16A | 5.1 |
| S16K | 5.1 |

Table 2. Kinetic parameters of the wild-type and mutant hydroxylases

| Enzyme | $K_m$, $\mu M$ | | $V_{max}$, $\mu mol \cdot min^{-1} \cdot mg^{-1}$ | $BH_4$ / $6MPH_4$ ratio* |
|---|---|---|---|---|
| | $BH_4$ | Phe | | |
| Wild type | 2.5 | 200.0 | 0.38 | 0.077 |
| S16E | 3.4 | 183.0 | 1.15 | 0.221 |
| S16Q | 4.4 | 217.0 | 0.51 | 0.098 |
| S16S(*P*) | 4.6 | 187.0 | 1.25 | 0.25 |
| S16N | 3.6 | 287.0 | 0.45 | 0.09 |
| S16D | 2.8 | 266.0 | 0.78 | 0.156 |
| S16A | 2.5 | 288.0 | 0.32 | 0.0627 |
| S16K | 2.7 | 254.0 | 0.62 | 0.121 |

Initial velocities were determined as described in *Materials and Methods.*

*The ratio of activity with the natural cofactor $BH_4$ to that with the synthetic cofactor $6MPH_4$ provides a measure of the state of activation of the enzyme (see *Discussion*).

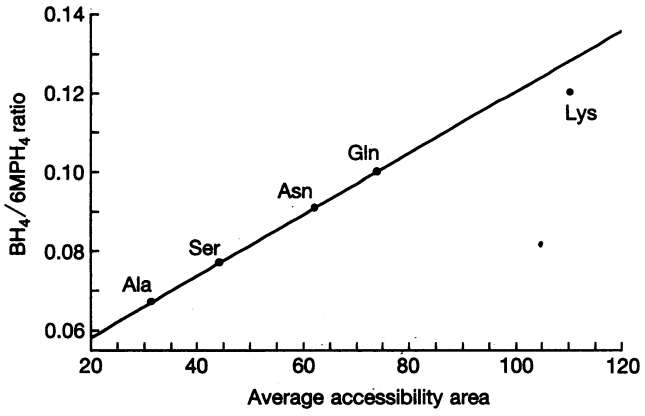


FIG. 3. Plot of the effect of molecular volume (shown as average accessibility area in $Å^2$) of alanine, serine, asparagine, glutamine, and lysine residues against the $BH_4/6MPH_4$ activity ratio (see footnote to Table 2 and *Discussion*) of the various substitution mutants.

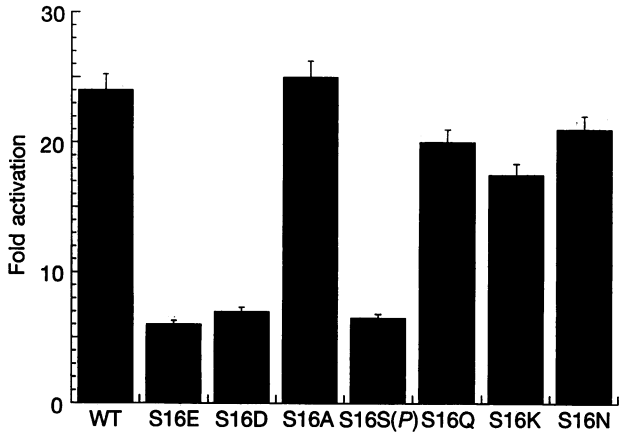


FIG. 4. Effects of lysolecithin on the catalytic activity of the wild-type (WT), phosphorylated wild-type [S16S(*P*)], and mutant hydroxylases. The reactions were followed spectrophotometrically as described in *Materials and Methods*. Results are the means and SEs from three experiments.

volume, however, is more modest than that resulting from the introduction of a negative charge.

**Effects of Lysolecithin on PAH Mutants and the Phosphorylated Enzyme.** It was of interest to determine whether activation by lysolecithin and activation by phosphorylation are independent or related phenomena. The wild-type, phosphorylated, and mutant forms of the hydroxylase showed different degrees of activation by lysolecithin (Fig. 4). The phosphorylated hydroxylase was stimulated 7.5-fold when assayed in the presence of 1 mM lysolecithin, whereas the wild-type enzyme was stimulated 24-fold. This result is comparable to the activation previously reported for the recombinant wild-type (13) and native (21) enzyme. Moreover, the mutants carrying single negative charges, S16E and S16D, were stimulated by lysolecithin to the same extent as the phosphorylated enzyme. The mutants S16A, S16Q, S16N, and S16K were activated by lysolecithin to about the same extent as the wild-type hydroxylase. These results are coherent with the previous conclusion that activation by phosphorylation and by lysolecithin are not additive, a finding that suggests that these modes of activation affect the same part of the molecule (6). The data in Fig. 5 extend this conclusion even to the modest activating effect due to the introduction of bulky amino acids at Ser-16. The state of activation of the various substitution mutants is shown to be inversely proportional to the stimulation by lysolecithin, an indication that the higher the state of activation, the lower the extent of activation by the phospholipid.

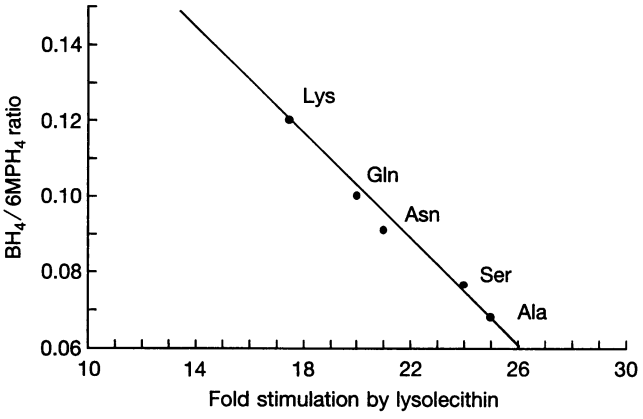


FIG. 5. Plot of $BH_4$/6$MPH_4$ activity ratio of various substitution mutants and wild-type PAH (Ser) against the degree of activation by lysolecithin.

## DISCUSSION

PAH is phosphorylated by PKA at a single position, Ser-16 (4). Phosphorylation of the hydroxylase has been shown to increase its activity 2- to 4-fold (21). Replacement of Ser-16 with a glutamate residue can stimulate the hydroxylase activity to the same extent as that seen after phosphorylation, suggesting that activation by phosphorylation results from introducing a negative charge at this position (12). These earlier results were rather preliminary, however, and did not include determination of $K_m$ or $V_{max}$ values of the mutant enzymes.

To more fully characterize the effects of various substitutions at Ser-16, we have used site-directed mutagenesis to replace this residue with the negatively charged amino acids, glutamate and aspartate, the polar uncharged amino acids glutamine and asparagine, and the positively charged amino acid lysine; the small nonpolar amino acid alanine served as a control. The wild-type and mutant enzymes were purified to homogeneity, and their kinetic characteristics and the effects of lysolecithin were studied. Activation of PAH by phosphorylation (21) and by other modes (23, 24) is fully expressed in the presence of $BH_4$, but not in the presence of the synthetic cofactor 6$MPH_4$. Since the activity of the hydroxylase measured in the presence of 6$MPH_4$ reflects only the intrinsic catalytic activity of the enzyme, the ratio of hydroxylase activity in the presence of $BH_4$ to that in the presence of 6$MPH_4$ serves as an index of the state of activation of the enzyme (25). Mutants carrying the negatively charged residue glutamate, aspartate, or phosphoserine in place of Ser-16 show an increase in this ratio (Table 2), suggesting that introduction of negative charge(s) at Ser-16 can stimulate the hydroxylase catalytic activity 2- to 4-fold. The present results, therefore, confirm and extend those obtained by Citron *et al.* (12), who studied the effect of replacing Ser-16 with glutamate (21).

The activating effect resulting from the substitution is due exclusively to increases in $V_{max}$; there are no consistent changes in the $K_{max}$ values for either phenylalanine or $BH_4$ (Table 2). An additional, more subtle activating effect appears to correlate with the volume of the amino acid at position 16 (Fig. 3). This relationship can adequately explain the otherwise puzzling and unexpected activating effect of substitution of lysine for Ser-16. The finding that the activating effect due to this last substitution appears to be somewhat less than would have been expected from its size may reflect a deactivating effect of the positively charged ε-amino group of lysine.

As previously reported, lysolecithin can further stimulate the phosphorylated enzyme, but the stimulation is much less than that seen with the native enzyme (21). Fig. 4 confirms these earlier results with the phosphorylated recombinant enzyme and shows that the phospholipid stimulates the mutants with negatively charged substituents (S16E and S16D) to about the same lesser extent as it does the phosphorylated enzyme, whereas the mutants bearing uncharged replacements are stimulated to the same extent as the wild-type enzyme. These results show that the activating effects due to the introduction of negative charge(s) and those due to lysolecithin are not additive. Rather, the data are consistent with the idea these different modes of activation are not independent but are on a common pathway. In further support of this notion, Fig. 5 shows that the extent of activation by lysolecithin is inversely proportional to the state of activation of the substitution mutants.

The present finding that replacement of Ser-16 with negatively charged residues activates the hydroxylase is in agreement with the proposal by Fisher and Kaufman (6) that the regulatory domain contains a peptide sequence that acts as an internal inhibitor of catalytic activity. This proposal was based on the observation that limited proteolysis of the enzyme, as well as interaction with certain phospholipids, led to marked activation. The activation was postulated to be due to the

irreversible removal and the reversible displacement, respectively, of the putative inhibitory polypeptide chain (6). Subsequently, it was shown that the region that exerts negative control over the catalytic activity is the regulatory domain located in the N-terminal third of the molecule (26). The observation that phosphorylation of Ser-16 in this domain activates the enzyme could be accounted for by this model, since the introduction of an additional negative charge in this region can be visualized as partially displacing the inhibitory peptide from the catalytic domain through electrostatic repulsion.

The present results provide added support for this model of the regulation of PAH and for the proposal that electrostatic interactions are responsible for the activation by phosphorylation. Thus, replacement of Ser-16 with a negatively charged amino acid closely mimics the activation effect of phosphorylation of this residue, whereas replacement by a size-matched uncharged amino acid has a much smaller effect. That these neutral molecules can modestly activate the enzyme and that the activation appears to be proportional to their size (Fig. 3) suggests that some displacement of the regulatory domain, with consequent activation, can result from steric effects of the bulky side chains. A full assessment of the validity of this last notion will require further study.

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