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# ELECTROSTATIC ACTIVATION OF RAT PHENYLALANINE HYDROXYLASE

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**SUMMARY:** The conversion of phenylalanine to tyrosine is accelerated approximately five fold by phosphorylation of the enzyme which catalyzes this step, phenylalanine hydroxylase. To gain a clearer understanding of the mechanism of this activation, we have applied site-directed mutagenesis to specifically modify a clone of the hydroxylase at the phosphorylation site, the serine at position 16. We converted this serine residue to alanine and to glutamic acid. The wild-type and mutant proteins were purified and the activation states of the enzymes were examined with respect to the single phosphorylation site at position 16. Substitution of $Ser^{16}$ with a negatively charged Glu residue resulted in activation of the enzyme, whereas substitution with an uncharged Ala residue did not. These results indicate that activation of the native enzyme by phosphorylation is due to the introduction of a negative charge, and suggest involvement of electrostatic interactions.

Phenylalanine hydroxylase catalyzes the conversion of phenylalanine to tyrosine and functions as part of a multicomponent system (1-3). The hydroxylation reaction is the rate-limiting step in the major catabolic pathway for the degradation of phenylalanine to $CO_2$ and water (4). Genetic defects resulting in the loss of hydroxylase activity are characterized by marked elevations in blood phenylalanine levels and, if untreated, can cause severe mental retardation (5). The hydroxylase, which is present in the liver and kidney, must respond to a widely varying dietary intake of phenylalanine. To insure that appropriate levels of phenylalanine are metabolized, phenylalanine hydroxylase has evolved with multiple regulatory mechanisms. These include long term and short term tactics that may be important for phenylalanine homeostasis. The balance

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The abbreviations used are: $BH_4$, (6R)-5,6,7,8-tetrahydrobiopterin; $6MPH_4$, 6-methyl-5,6,7,8-tetrahydropterin.

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between phenylalanine levels that are too low for efficient protein synthesis and levels that are too high for normal central nervous system development seems sufficiently important for nature to have evolved a variety of overlapping regulatory mechanisms for this reaction.

Phenylalanine hydroxylase of humans and rats is a 453 amino acid protein with a subunit molecular weight of 52,000. Rapid changes in the rate of hydroxylation can result from phenylalanine-mediated activation of the enzyme (6-9), and deactivation by its pterin cofactor (10). In addition, the hydroxylase can be activated by phospholipids (11) and by posttranslational modification by cAMP-dependent protein kinase (PKA) (12, 13). This kinase phosphorylates each subunit of phenylalanine hydroxylase at $Ser_{16}$ (14) at a consensus site for cAMP-protein kinase (15).

Phosphorylation plays a role in activation of phenylalanine hydroxylase *in vitro* (13) and *in vivo*. Administration of glucagon to rats was shown to increase the level of phosphorylation of the hepatic enzyme with a concomitant increase in the rate of hydroxylation (16). Moreover, activation of the hydroxylase by its substrate is synergistic with activation by phosphorylation. Thus, in the rat, phenylalanine has been shown to markedly stimulate the phosphorylation of the hepatic enzyme (17).

In the present study, we have investigated the nature of the activation of phenylalanine hydroxylase resulting from the PKA-catalyzed phosphorylation of $Ser_{16}$. To determine whether phosphorylation at this residue is due to the introduction of a negative charge in this region, $Ser_{16}$ was replaced with a glutamic acid residue. As a neutral control, $Ser_{16}$ was also replaced with an alanine residue. The mutagenesis was performed with a clone of the rat liver phenylalanine hydroxylase coding sequence that expresses an enzyme with kinetic parameters similar to the enzyme isolated from rat liver (18).

## MATERIALS AND METHODS

**DNA Manipulations.** Plasmid purifications, restriction digestions, ligations, and transformations, were performed according to standard procedures (19, 20). The plasmid pLNC209 is a bacterial plasmid clone that expresses rat phenylalanine hydroxylase (18). Charged nylon membranes for hybridization were Colony/Phage screening membrane from New England Nuclear. Restriction endonucleases and DNA modifying enzymes were obtained from New England Biolabs, Bethesda Research Laboratories, Promega, and United States Biochemicals. [$\alpha^{32}$P]dATP and [$\gamma^{32}$P]ATP were obtained from New England Nuclear. The *E. coli* host strain was DH5 (F- *endA1 hsdR17 supE44 thi1 recA1 gyrA96 relA1 Δ(lacZYA-argF)U169)* (21).

**Mutagenesis.** The mutagenic primer was phosphorylated with T4 polynucleotide kinase. Plasmid pLNC209 was divided and double-digested with *Eco*RI and *Eco*RV and separately with *Nru*I (Fig. 1). Following enzyme inactivation, 500 ng of each DNA was mixed with 50 pmol of oligomer in 60 µl total volume. The DNAs were denatured at 100° for 5 min, and annealed by cooling to 30° for 30 min, then 4° for 30 min, and finally 0° for 10 min. The remaining single-stranded gaps were filled in with the Klenow fragment of DNA polymerase I and then sealed with T4 DNA ligase at 12.5° for 17 h in 70 µl total volume with 150 µM each dNTP, 300 µM ATP, 2 U of Klenow, and 1 U (Modrich) of ligase. Approximately 300 transformants were obtained per µl of this reaction.

**DNA Hybridization.** Colony hybridizations (22, 23) were performed by transforming DH5 cell with putative mutant plasmids, incubation at 37° overnight, chilling to 4° for 2 hours, and transfer of colonies for 1 min to charged nylon. In volumes of 10 ml per filter, the bound DNA was denatured for 20 s in 0.1 N NaOH, 1.5 M NaCl, neutralized with two 20 s washes in 0.2 M Tris HCl, pH 7.5, blotted on GB002 filter paper, crosslinked for 2 min at 1200 µW/cm$^2$ at 254

nm, prewashed 2 h in 50 mM Tris HCl, pH 8.0, 1 M NaCl, 1 mM EDTA, 0.2% sodium dodecylsulfate (SDS), 2 h, prehybridized 2 h in 0.5 M sodium phosphate pH 7.2, 1 mM EDTA, 10 mg/ml bovine serum albumin, 7% SDS, and hybridized 17 h with 2 x $10^6$ dpm of oligomer O35 (labeled to $10^6$ dpm/pmol with [$\gamma^{32}$P]-ATP and T4 polynucleotide kinase) per filter at 54°, washed in 100 ml/filter, two times 5 min each at 23° with 2 X standard saline citrate (SSC=0.15 M NaCl, 0.015 M sodium citrate), 30 min at 50° with 2 X SSC, 1% SDS, and 10 min at 45° with 1 X SSC. After autoradiography, positive colonies were isolated and preserved.

**Colony PCR.** The polymerase chain reaction was used directly on cells scraped from LB-ampicillin plates to amplify with a primer diagnostic for the S16A mutation. One to four colonies were placed in a 0.5 ml microfuge tube with 20 µl of 1 X PCR reaction mix (1.5 mM $MgCl_2$, 10 mM Tris HCl, pH 8.3, 50 mM KCl, 10 µg/ml gelatin), lysed at 99° for 2 min, cooled momentarily to 90°, and brought to 100 µl with the same reactants plus 0.2 mM each dNTP, 0.5 µM oligo O104, 0.5 µM O94, and 0.5 U Taq polymerase (Amplitaq, Perkin Elmer). The amplification consisted of 40 cycles of denaturation at 94° for 1 min, annealing at 60° for 1 min, and extension at 72° for 1.5 min followed by a final extension at 72° for 7 min and cooling to 4°.

**DNA Sequence Analysis.** The dideoxy sequencing protocol (24) was performed on double-stranded DNA templates with modified T7 DNA polymerase (United States Biochemicals) and the products were analyzed on an EG&G (Natick, MA) Acugen automated DNA sequencer. University of Wisconsin Genetics Computing Group software was used to manipulate the DNA and derived protein sequences (25).

**Bacterial Cultures.** Bacterial growth medium (26), LB (0.5% NaCl, 1% tryptone (DIFCO), 0.5% yeast extract (DIFCO)), was supplemented as required with ampicillin (100 µg/ml), isopropyl-β-D-thiogalactopyranoside (0.5 mM), and ferrous sulfate (0.1 mM). *E. coli* cells harboring recombinant plasmids were grown in 300 liters of LB-ampicillin-iron at 37° for six hours after addition of a 1% inoculum and isopropyl-β-D-thiogalactopyranoside.

**Protein Purification.** The wild-type and mutant phenylalanine hydroxylases were purified from the bacterial cells by sonication, ammonium sulfate precipitation, phenyl-Sepharose chromatography, DEAE-cellulose chromatography, and concentration by Amicon filtration (27, 28) with the previously described modifications (18), from thirty-five grams (wet weight) of *E. coli*. Protein was quantitated with the Pierce Bradford assay. Purified enzymes were examined on SDS-10% polyacrylamide gels (29).

**Enzyme Assays.** The enzyme assays included sheep liver dihydropteridine reductase purified through a calcium phosphate gel (30), catalase from Boehringer -Mannheim, $BH_4$ and $6MPH_4$ from Dr. B. Schircks (Jona, Switzerland), and L-phenylalanine and NADH obtained from Sigma. The assays were conducted at 25° in 1 ml reactions and contained 0.1 M potassium phosphate, pH 6.8, excess dihydropteridine reductase, 100 µg/ml catalase, 1.0 mM phenylalanine, 128 µM NADH, 33 µM $BH_4$, and 6.5-35 µg/ml phenylalanine hydroxylase. When $6MPH_4$ was used in place of $BH_4$, the same reaction mixtures were used except for the quantities of phenylalanine (5.0 mM), $6MPH_4$ (275 µM), and phenylalanine hydroxylase (1.3-6 µg/ml). Initial rates were monitored spectrophotometrically at 340 nm. In this assay, the rate of hydroxylation is measured as the rate of the dihydropteridine reductase-catalyzed reduction by NADH of quinonoid dihydropterin (produced during the hydroxylation reaction) to tetrahydrobiopterin (27).

## RESULTS AND DISCUSSION

To covalently modify the phosphorylation site of phenylalanine hydroxylase, we applied gapped duplex mutagenesis to convert the serine at position 16 to an alanine or a glutamic acid. The recombinant rat phenylalanine hydroxylase clone which expresses wild-type hydroxylase was double digested with restriction endonucleases *Eco*RI and *Eco*RV and also singly digested with *Nru*I in a separate tube. Following inactivation of the restriction enzymes, the DNAs were mixed, denatured and annealed so that heterodimers could form between plasmid linearized with *Nru*I, and the fragments produced by the *Eco*RI plus *Eco*RV digestion (see Figure 1). Plasmid pLNC209 (6778 bp) has an *Eco*RI site at 136, an *Eco*RV site at 318, and an *Nru*I site at position 6058. Although many combinations were formed, a significant percentage of the products were expected

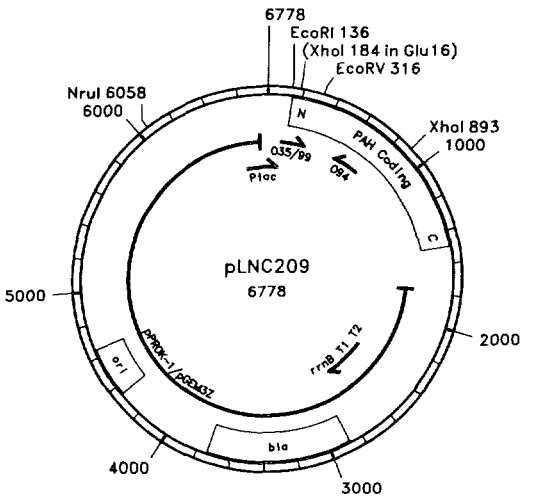


Fig. 1. **Gapped duplex mutagenesis of rat phenylalanine hydroxylase clone.** The full hydroxylase coding sequence is present and optimally positioned downstream from a ribosome binding site and a promoter (Ptac) region. The insert is followed by a transcription terminator (rrnB T1 T2). The vector is a composite molecule of pPROK-1 and pGEM3Z sequence (18) and harbors a colE1 replication origin (ori) and an ampicillin resistance marker (bla). Partially single-stranded DNA molecules for mutant primer annealing were produced with the unique restriction sites for *Eco*RI, *Eco*RV, and *Nru*I. The locations of the mutagenic oligomers (O35/O94) are depicted along with the downstream amplification primer (O94) which was used for diagnostic amplification of the Serine–>Alanine substitutions. The *Xho*I and *Pvu*II sites facilitated the evaluation of putative Serine–>Glutamic acid substitution mutants.

to form an open circle that was predominantly double-stranded with a small single-stranded gap that would be available for hybridization to a mutagenic oligonucleotide primer. Following hybridization to either oligomer O35 or O99 (see Figure 2), the gaps were filled in with DNA polymerase I and sealed with DNA ligase. These composite DNA molecules were then used to transform *E. coli* cells and transformants were screened for the presence of the mutations. Approximately 1-5 % of the ampicillin resistant colonies harbored mutated plasmids.

The mutant produced by O35 was screened by colony hybridization (22) with the identical mutant 24 base oligonucleotide which did contain three base changes. These base substitutions were also initially selected to introduce a new *Xho*I recognition site into the plasmid to aid screening the candidates by restriction analysis with *Xho*I which also cleaves at 893. The mutants produced by O99 were initially detected by polymerase chain amplification with oligomers O104 and O94 (see Figures 1 and 2). Wild-type sequences would not produce a product due to the single mismatch at the 3' end of O104. These mutations were all confirmed by double-stranded DNA sequence analysis and one of each mutant and also the wild-type parent plasmid were then expressed in large-scale cultures and the phenylalanine hydroxylases produced were purified to homogeneity (Fig. 3) and examined with respect to their activation state.

In order to determine whether activation of phenylalanine hydroxylase by PKA-mediated phosphorylation of $Ser^{16}$ is due to the introduction of the negatively charged phosphate moiety, the serine at this position was replaced with the negatively charged glutamic acid residue; substitution with alanine served as a neutral control.

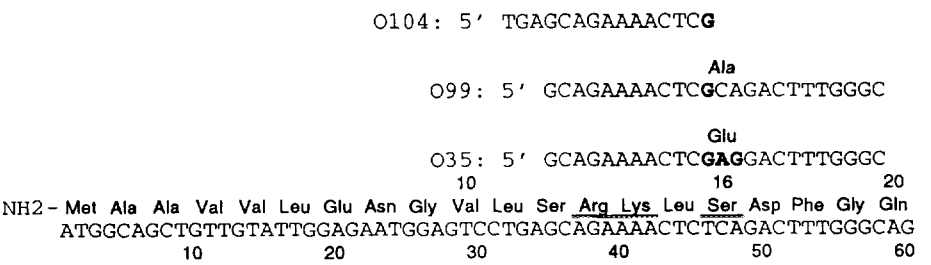


Fig. 2. **The DNA and protein sequence spanning the phosphorylation site.** The mutagenic primers (O35 and O99) are depicted along with the DNA and protein substitutions produced. The Glu substitution mutants were also detected, by hybridization, with the mutagenic oligomer O35. Oligomer O104 was added as the 5' primer in DNA amplifications to detect the single base change required for the alanine substitution. The consensus sequence for cAMP-dependent protein kinase (PKA) phosphorylation is underscored.

To assess the effect of these substitutions on the state of activation of the enzyme, hydroxylase activity was measured in the presence of $BH_4$ and the synthetic cofactor analogue, $6MPH_4$. Since activation by phosphorylation (12), like most other modes of activation of the enzyme (31), is fully expressed in the presence of $BH_4$ but not in the presence of synthetic cofactors like $6MPH_4$ or $DMPH_4$, the ratio of hydroxylase activities in the presence of $BH_4$ and $6MPH_4$ (or $DMPH_4$) serves as a measure of the state of activation of the enzyme (32). Essentially, the activity in the presence of $6MPH_4$ is a measure of the amount of enzyme, independent of its state of activation. As can be seen from the results in Table 1, the $Ser^{16}$->Glu substitution activates the enzyme 3-fold, about the same as the 2.5-fold phosphorylation-mediated activation previously reported for the wild-type recombinant enzyme (18).

Activation by phosphorylation of $Ser^{16}$ could result from either of two mechanisms. First, the hydroxyl group of this serine residue in the unmodified enzyme could be involved (e.g., *via* hydrogen bonding) in keeping the enzyme in a low activity conformation. In this case, replacement of $Ser^{16}$ with an Ala residue should activate. Alternatively, the low activity

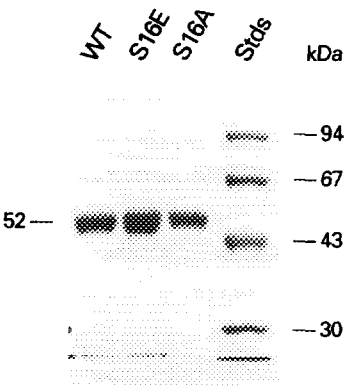


Fig. 3. **The purified wild-type and mutant phenylalanine hydroxylases.** The wild-type, Ser→Glu, and Ser→Ala substituted enzymes are shown, after purification, on an SDS-denaturing polyacrylamide gel.

Table 1. Activity of rat phenylalanine hydroxylase mutant enzymes. The $6MPH_4/BH_4$ ratio is indicative of the activation state of the enzyme. Lower values correspond to activated hydroxylase.

| Enzyme | Cofactor | | |
|---|---|---|---|
| | $BH_4$ (μmol/min/mg) | $6MPH_4$ (μmol/min/mg) | $6MPH_4/BH_4$ (ratio) |
| Wild-Type | 0.36 | 4.3 | 12 |
| $Ser^{16}\rightarrow Ala$ | 0.25 | 6.0 | 24 |
| $Ser^{16}\rightarrow Glu$ | 1.47 | 5.9 | 4 |

conformation could be stabilized by the interaction of positively and negatively charged regions of the protein. In this case, replacement of the $Ser^{16}$ with the negatively charged Glu might mimic the effects of phosphorylation of $Ser^{16}$ and lead to activation, whereas the Ala substitution would not.

The $Ser^{16}$–>Ala substitution, unexpectedly, proved not to be a neutral modification, but rather resulted in some deactivation of the enzyme. Although the reason for this lower state of activation is not known with certainty, it may be due to the presence in the wild-type recombinant enzyme of a small amount of phosphate (approximately 0.15 mol/mol subunit) (18). If that phosphate is present on $Ser^{16}$, the recombinant wild-type enzyme would likely be in a somewhat activated state. In that case, replacement of the partially phosphorylated $Ser^{16}$ with alanine would be expected to lead to partial deactivation.

The finding that the $Ser^{16}$–>Glu substitution activates the hydroxylase is in accord with the "internal inhibitor" model for the structural organization of the enzyme, originally proposed to account for activation by phospholipids and by limited proteolysis and phosphorylation (11, 12). Based on these findings, it was proposed that in its native state, the activity of the hydroxylase is negatively regulated by an inhibitory polypeptide and that activation involves the irreversible removal (e.g. proteolysis) or the reversible displacement (e.g. by the action of certain phospholipids or by the introduction of a negative charge through phosphorylation) of the inhibitory region from the catalytic domain, which thereby activates the enzyme. It was demonstrated that activation of the hydroxylase by limited proteolysis removes the site of phosphorylation (12) and that this site is in the regulatory domain which is located toward the N-terminal region of the enzyme (33). The present results, therefore, strengthen the conclusion, derived from our earlier phosphorylation studies, that the inhibitor effect of the regulatory domain involves electrostatic interaction between these two regions of the enzyme. Future work should elucidate the precise molecular interactions important in hydroxylase regulation.

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