Phosphorylation and Mutations of Ser\textsuperscript{16} in Human Phenylalanine Hydroxylase

KINETIC AND STRUCTURAL EFFECTS\textsuperscript{a}

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Phosphorylation of phenylalanine hydroxylase (PAH) at Ser\textsuperscript{16} by cyclic AMP-dependent protein kinase is a post-translational modification that increases its basal activity and facilitates its activation by the substrate L-Phe. So far there is no structural information on the flexible N-terminal tail (residues 1–18), including the phosphorylation site. To get further insight into the molecular basis for the effects of phosphorylation on the catalytic efficiency and enzyme stability, molecular modeling was performed using the crystal structure of the recombinant rat enzyme. The most probable conformation and orientation of the N-terminal tail thus obtained indicates that phosphorylation of Ser\textsuperscript{16} induces a local conformational change as a result of an electrostatic interaction between the phosphate group and an Arg\textsuperscript{13} as well as a repulsion by Glu\textsuperscript{280} in the loop at the entrance of the active site crevice structure. The modeled reorientation of the N-terminal tail residues (Met\textsuperscript{1}–Leu\textsuperscript{15}) on phosphorylation is in agreement with the observed conformational change and increased accessibility of the substrate to the active site, as indicated by circular dichroism spectroscopy and the enzyme kinetic data for the full-length phosphorylated and nonphosphorylated human PAH. To further validate the model we have prepared and characterized mutants substituting Ser\textsuperscript{16} with a negatively charged residue and found that S16E largely mimics the effects of phosphorylation. The phosphorylated (at Ser16) enzyme requires a lower concentration of substrate for its activation (15, 16). It appears that these two mechanisms act synergistically also in vivo and that L-Phe promotes the phosphorylation and activation of PAH in rat liver (13, 17). However, the molecular mechanism of this interdependence is not well understood (18, 19) and has not been explained by the crystal structure analyses of the phosphorylated (at Ser\textsuperscript{16}) AC24-truncated dimeric form of rat PAH (rPAH) (9). Phosphorylation of the human enzyme results in a mobility shift on SDS-PAGE (20) that is also observed when hPAH is expressed in Escherichia coli (16, 21) and in the \textit{in vitro} transcription-translation system (5, 22). The enzyme expressed in the latter system is recovered as a double band on SDS-PAGE, corresponding to the phosphorylated (~51 kDa) and nonphosphorylated (~50 kDa) forms. Furthermore, we have previously shown by Fourier transform infrared spectroscopy that phosphorylation of the isolated recombinant N-terminal regulatory domain (residues 2–110) results in an apparent increase in the content of \(\alpha\)-helical structure (23). In the present work we have extended these studies, including molecular modeling by the anchor grow method using the program Dock 4.0 (24), and the possible conformations of the 18-residue N-terminal tail have been estimated for the nonphospho-

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\textsuperscript{2} The abbreviations used are: PAH, phenylalanine hydroxylase; BH\textsubscript{4}, (6R)-erythro-5,6,7,8-tetrahydrobiopterin; hPAH, human phenylalanine hydroxylase; PKA, cyclic AMP-dependent protein kinase; rPAH, rat phenylalanine hydroxylase; wt-hPAH, wild-type hPAH.
rylated and phosphorylated states. The modeled conformations have given a structural explanation of the changes in enzyme kinetic properties and conformation observed as a result of phosphorylation. To validate this modeled structure we substituted Ser16 by residues with negatively charged (Glu and Asp) and neutral (Ala) side chains. In addition, we have studied the phosphorylated states. The biochemical data support the structural domains considered to be involved in electrostatic interactions shown in Table I. The authenticity of the mutagenesis was verified by Plus (University of California at San Francisco) (25), and a grid of 45 Å face of this domain was calculated by the DMS program under Midas-Plus (Nonlinear Dynamics Ltd). The reaction was initiated by adding 500 μM MgATP and the extent of phosphorylation was measured by SDS-PAGE (mobility shift) and/or by 32P autoradiography using γ32P]ATP (Amersham Biosciences). At these conditions ~1 mol of phosphate was incorporated per mol isolated hPAH subunit (16). The samples of nonphosphorylated wt-hPAH and mutants utilized in this study were treated at identical conditions as the phosphorylated enzymes, except for the absence of kinase during assay.

**Table I**

| Mutant | Sense | Sequence |
|--------|-------|---------|
| S16A   | Forward | 5′-TTG-GGC-AGG-AAA-CTC-GCA-GAC-TTT-GGA-CAG-3′ |
| S16A   | Reverse | 5′-CTG-TCC-AAA-GTC-GTC-GAG-TTT-CCT-GGC-CAA-3′ |
| S16D   | Forward | 5′-TTG-GGC-AGG-AAA-CTC-GAT-GAG-TTT-CAG-3′ |
| S16D   | Reverse | 5′-CTG-TCC-AAA-GTC-ATC-GAG-TTT-CCT-GGC-CAA-3′ |
| S16E   | Forward | 5′-TTG-GGC-AGG-AAA-CTC-GAA-GAC-TTT-GGA-CAG-3′ |
| S16E   | Reverse | 5′-CTG-TCC-AAA-GTC-TTC-GAG-TTT-CCT-GGC-CAA-3′ |
| R13A   | Forward | 5′-CCA-GGC-TTG-GGC-AAA-CTC-TCT-GAC-3′ |
| R13A   | Reverse | 5′-GCC-CAA-GAG-TTT-GGA-CAG-3′ |
| E353A  | Forward | 5′-CTG-TCA-TCC-TTT-GCT-GTA-CAG-TAC-TGC-3′ |
| E353A  | Reverse | 5′-GCC-GTA-CTG-TAA-TGC-ACC-AAA-GGA-TGA-CAG-3′ |
| E381A  | Forward | 5′-TAC-ACT-GTC-AGG-CTG-TTC-CAG-CCC-CTG-TAT-TAC-3′ |
| E381A  | Reverse | 5′-GTA-ATA-CAG-GGC-GGG-TCC-GAA-IGC-GCT-GAG-AGT-GTA-3′ |

**EXPERIMENTAL PROCEDURES**

**Molecular Modeling**—Docking and preparation of structures was performed on a SGI Octane workstation. The DOCK 4.0.1 suite of programs (University of California at San Francisco) (24) was used to fit the conformers of the N-terminal peptide sequence Met1 into rigid segments containing the largest set of adjacent atoms separated by nonrotatable bonds, whose position is optimized during each step. The coordinates for Gly19 (MAAV-VLENGVLSRLSDFG) into the crystal structure of the dimeric rPAH (9), including the regulatory domain (from Gly19), the catalytic domain, and the dimerization motif (up to Thr44) were used. The solvent-accessible surface of this domain was calculated by the DMS program under Midas-Plus (University of California at San Francisco) (25), and a grid of 45 Å × 29 Å × 36 Å was constructed in which ion was included as a sphere of the correct radius and without including crystallographic water molecules. The grid is used by DOCK to evaluate the steric boundary of the protein-detergent complex) have different slopes and a common intercept at the y axis, whereas proteins differing only in the electrical charge have identical slopes with different y axis intercepts (30, 32).

**Limited Tryptic Proteolysis**—The various forms of hPAH (0.1 μg/μl) were incubated with Np-tosyl-l-phenylalanine chloromethyl ketone-treated trypsin from bovine pancreas (Sigma) at various protease:PAH ratios, i.e., 0.002:1, 0.005:1, 0.01:1, and 0.1:1, in a final volume of 45 μl at 30 °C for 20 min. After quenching the reaction with SDS denaturation buffer by heating for 3 min at 95 °C, the samples were analyzed by SDS-PAGE.

**Assay of PAH Activity**—PAH activity was assayed at 25 °C with a standard reaction mixture (final volume, 50 μl) of 10 μl ferrous ammonium sulfate, 1 mM L-Phe, 5 mM dithiothreitol, and 75 μM BH4, 0.04 μg/μl catalase, and 0.05% (w/v) bovine serum albumin, and 1 μg of the various hPAH forms in 100 mM Na-HEPES, pH 7.0, as described (21). Two types of reactions were performed: (i) the enzyme was preincubated for 4 min at 25 °C in a mixture containing buffer, L-Phe, and catalase, then Fe(II) was added, and after another 1-min incubation, the reaction was started by the addition of BH4 (L-Phe activated enzyme) and (ii) the reaction was performed as above, except that L-Phe is added together with BH4 (nonactivated enzyme). The reaction was stopped after 30 s with 1% (v/v) acetic acid in ethanol. The amount of L-Tyr was measured by high pressure liquid chromatography and fluorimetric detection (12, 21). The kinetic parameters were determined using variable concentrations of the substrate (0–5 mM L-Phe with 75 μM BH4) and of the cofactor (0–500 μM BH4 with 1 mM L-Phe) and were calculated by nonlinear regression analysis of the experimental data using the program Enzfitter (Elsevier-Biosoft).

**Circular Dichroism**—CD analyses were performed at 25 °C on a Jasco J-810 spectropolarimeter equipped with a Jasco 423S Peltier
element. The samples (10 μM subunit of hPAH forms) were prepared in 20 mM sodium phosphate, 50 mM KF, pH 7.0, with stoichiometric amounts of ferrous ammonium sulfate. Wavelength scans were taken between 185 and 300 nm using quartz cells with path lengths of 0.1 and 0.5 cm. The data were analyzed with the standard analysis program provided with the instrument. The amount of secondary structure elements was estimated by the CDNN program that applies a neural network procedure.

RESULTS

Modeling the Conformational Changes Induced by Phosphorylation—In the crystal structure of the dimeric C-terminal truncated form of rPAH (Protein Data Bank code 1PHZ) (9), no electron density was observed for the 18-residue N-terminal tail. Therefore, to obtain some information on the structure of the full-length enzyme and on the conformational changes induced by phosphorylation of Ser16, we have created a modeled structure of the N-terminal tail by docking the peptide sequence into the crystal structure of rPAH, using DOCK with the anchor grow method (24). Using the coordinates for Gly19 as an anchor for growing the remaining 18 N-terminal residues, the most probable conformations for the 19-residue N-terminal tail was estimated for the nonphosphorylated and phosphorylated forms. The top scoring structures resulting from the molecular docking are shown in Fig. 1 for the nonphosphorylated (Fig. 1A) and the phosphorylated forms (Fig. 1B) (see also the supplemental material). Whereas the residues 16–18 could be superimposed for the two forms, phosphorylation resulted in a significantly different conformation/orientation of residues 1–15. Furthermore, Arg13 was found to change its electrostatic interactions from Glu353 and Glu381 in the nonphosphorylated enzyme (Fig. 1C) to the phosphate group of Ser16 (Fig. 1D). Although the nonphosphorylated tail establishes interactions with residues on both sides of the entrance to the active site crevice structure, leading to a relatively “closed” conformation of this structure, the phosphorylated tail is reoriented along one side of the channel (Table II), resulting in a more open active site conformation. This change in orientation would allow for the proposed facilitated access of the substrate (9) to its binding site close to the iron (34).

The electrostatic interaction between the phosphate group at Ser16 and Arg13 thus seems to be a key determinant for the different conformation adopted by the phosphorylated enzyme. To further validate this structural model and to get information on the molecular basis for the kinetic and conformational changes that occur in wt-hPAH on phosphorylation, we substituted Ser16 with acidic residues (using alanine as a control) and characterized the biochemical properties of the resulting enzyme forms.

Expression, Purification, and SDS-PAGE Analysis of Enzyme Forms of hPAH with Substitutions at Ser16—The mutants S16A, S16D, and S16E were expressed and purified at similar yields as those obtained for wt-hPAH, i.e. about 35 mg of fusion protein were obtained per liter of bacterial culture, and about 8–12 mg of pure tetrameric enzyme forms were recovered after the cleavage of the fusion protein by EKMaxTM. When wt-hPAH is phosphorylated on Ser16 by PKA, a mobility shift is observed on SDS-PAGE for the phosphorylated form (which appears at an apparent Mr of ~51 kDa) with respect to the nonphosphorylated form (~50 kDa) (16, 21) (Fig. 2A). A similar result was obtained when wt-hPAH expressed in the in vitro transcription-translation system was analyzed (Ref. 5 and data not shown). The relative mobilities (Rm values) on SDS-PAGE of the S16D and S16E mutants are also lower than that of the nonphosphorylated wt-hPAH, although the mobility shift was less pronounced than that observed for phosphorylated wt-hPAH (Fig. 2A). To get further information on the mobility shift observed for phosphorylated wt-hPAH, the shift was measured as a function of the pore size of the gel and analyzed by the Ferguson plot method (30, 31) using standardized gels with protein standards of known radii. From Fig. 2B it is seen that the difference in Rm values between the phosphorylated and nonphosphorylated subunits increased with decreasing pore size. These data indicate that the mobility shift results from a change in the effective Stokes radius of the SDS-denatured enzyme, as reported for other proteins (32). Analysis of the mobility shift of the mutant forms S16D and S16E relative to nonphosphorylated wt-hPAH or to the S16A mutant showed that the differences in the slope of the log (Rm) versus the acrylamide concentration plots were not significant. Attempts to study the mobility shift following phosphorylation under nondenaturing conditions was hampered by the fact that the

Fig. 1. Molecular docking of the 19-residue N-terminal segment into the crystal structure of a dimeric form of rat PAH. Representative (top score) structures of the nonphosphorylated (A) and phosphorylated (B) 19-residue N-terminal tail (shown as a ribbon representation of the backbone in black) docked into the structure of rat PAH monomer (Protein Data Bank code 1PHZ) (9), lacking the structure of the residues 1–18. The rest of the regulatory domain and the catalytic domain are shown as a representation of the electrostatic surface potential. The iron is shown in yellow. C and D, stereo representations of the nonphosphorylated (C) and phosphorylated (D) 19-residue N-terminal tail. Residues close to both Ser16 and Arg13 are shown as sticks. See text for details.
**Phosphorylation and Mutations of Ser**<sup>16</sup> **in hPAH**

**Table II**

| N-terminal residue | wt-hPAH | Phosphorylated wt-hPAH |
|--------------------|---------|-----------------------|
| Met<sup>a</sup>    | NC      | NC                    |
| Ala<sup>a</sup>    | NC      | NC                    |
| Ala<sup>a</sup>    | NC      | NC                    |
| Val<sup>a</sup>    | NC      | NC                    |
| Val<sup>a</sup>    | Asp<sup>145</sup>(L), Pro<sup>347</sup>(L)<sup>b</sup> | Glu<sup>276</sup>(R) |
| Leu<sup>a</sup>    | Tyr<sup>277</sup>(L) | Ser<sup>175</sup>(R) |
| Glu<sup>a</sup>    | NC      | Ser<sup>175</sup>(R), Pro<sup>344</sup>(R) |
| Asn<sup>a</sup>    | NC      | Thr<sup>380</sup>(R), Pro<sup>384</sup>(R) |
| Glu<sup>a</sup>    | Thr<sup>380</sup>(R), Glu<sup>381</sup>(R) | Ser<sup>175</sup>(R), Val<sup>175</sup>(R) |
| Leu<sup>a</sup>    | Thr<sup>380</sup>(R) | Val<sup>175</sup>(R) |
| Ser<sup>a</sup>    | Thr<sup>380</sup>(R) | Val<sup>175</sup>(R), Thr<sup>380</sup>(R), Glu<sup>381</sup>(R) |
| Arg<sup>a</sup>    | Glu<sup>353</sup>(R), Glu<sup>381</sup>(R) | Pro<sup>279</sup>(L), Asp<sup>145</sup> |
| Lys<sup>a</sup>    | Thr<sup>274</sup>(L), Pro<sup>383</sup>(L), His<sup>385</sup>(L), Glu<sup>260</sup>(L), Pro<sup>381</sup>(L), Gly<sup>266</sup>(R) | Gly<sup>346</sup>(R) |
| Leu<sup>a</sup>    | NC      | NC                    |
| Ser<sup>a</sup>    | NC      | NC                    |
| Asp<sup>a</sup>    | NC      | NC                    |
| Phe<sup>a</sup>    | NC      | NC                    |
| Gly<sup>a</sup>    | NC      | NC                    |

<sup>a</sup> NC, no contacts.

<sup>b</sup> The (R) and (L) correspond to location at the right and left side of the channel leading to the active site iron when looking towards the iron (same orientation as shown in Fig. 1), respectively.

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**Fig. 2.** SDS-PAGE of phosphorylated and nonphosphorylated wild-type and mutant forms of hPAH at Ser**<sup>16</sup>. A, SDS-PAGE of the isolated tetrameric forms (0.5 μg/lane). Lane 1, low molecular mass standard proteins; lane 2, wt-hPAH; lane 3, phosphorylated wt-hPAH; lane 4, S16A; lane 5, S16D; lane 6, S16E. B, Ferguson plot analysis of the mobility shift of hPAH on phosphorylation. 1 μg of phosphorylated (P) and nonphosphorylated wt-hPAH was applied in separated lanes in gels of 8, 10, and 13% (w/v) acrylamide. The slope for the linear regression was 0.0021.

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purified tetrameric enzyme does not show a unique band when subjected to native electrophoresis but rather a smear of multiple bands, probably related to the microheterogeneity caused by deamidation of several labile Asn residues in the subunit (35).

**Steady-state Kinetic Analysis**—As seen from Table III, the

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V<sub>max</sub> values obtained for the three mutant forms S16A, S16D, and S16E were similar to that of wt-hPAH when the enzymes were activated by preincubation with l-Phe. As expected (16), phosphorylation of hPAH resulted in a 1.4-fold increase in the basal activity (i.e. without l-Phe preincubation) as well as a higher apparent affinity for l-Phe. No significant difference in the degree of substrate activation or the kinetic parameters was measured for the S16A mutant form, as compared with the nonphosphorylated wt-hPAH, whereas the mutants with the negatively charged residues, notably S16E, revealed a higher basal activity and a lower [S]₀.₅ value for l-Phe, thus partially mimicking the phosphorylated form (Table III). The characteristic positive kinetic cooperativity of l-Phe binding to tetrameric PAH, with a Hill coefficient of h = −2 (1, 3, 21) was maintained both upon phosphorylation and substitution of Ser<sup>16</sup>. Neither phosphorylation of wt-hPAH (16) nor the mutations at Ser<sup>16</sup> had any significant effect on the K<sub>m</sub> value for the cofactor BH<sub>4</sub> (in the range of 29–35 μM).**

**Limited Tryptic Proteolysis**—We also studied the effect of phosphorylation and substitutions of Ser<sup>16</sup> on the susceptibility of the enzyme to limited tryptic proteolysis as a conformational probe. As seen from Fig. 3A, wt-hPAH (about 50 kDa for the subunit) generated several forms with molecular masses close to that of the full-length enzyme (about 49 kDa), in addition to truncated forms of molecular mass around 35 kDa, corresponding to the catalytic core structure (36). The rate of cleavage by limited tryptic proteolysis was decreased for the phosphorylated enzyme (Fig. 3, B and E). Although proteolysis of the mutant S16A gave results similar to those for the nonphosphorylated enzyme (Fig. 3, C and E), the substitution of Ser<sup>16</sup> with a negatively charged residue also protected against proteolysis by trypsin (Fig. 3, D and E, and data not shown for the S16D mutant).

**Circular Dichroism**—From the far UV CD spectra of nonphosphorylated and phosphorylated wt-hPAH and of the mutants S16A and S16E (Fig. 4), it is seen that the negative ellipticity in the 205–235-nm range increased on phosphorylation and on substitution of Ser<sup>16</sup> with negatively charged residues, compatible with an increase in the apparent α-helical content. Thus, using a standard analysis program, the α-helical content of nonphosphorylated hPAH and the mutant S16A was calculated to be 30.1 ± 1.3%, as compared with 38.7 ± 1.7% for the phosphorylated enzyme and 35.0 ± 2.4% for S16E when the spectra (185–250 nm) were simulated (33). A 33.1 ± 2.0% α-helical content was calculated for the S16D mutant form. The content of the α-helical structure for wt-hPAH estimated by CD is in agreement with that obtained by the same method for rPAH (31%) (37) and as determined in the crystal structure (−35%) (9).

**Expression, Purification, and Characterization of the Mutant Forms R13A, E353A, and E381A**—In addition to the site of phosphorylation, several residues reveal themselves as significant in maintaining the correct conformation and orientation of the N-terminal tail in the nonphosphorylated and phosphorylated hPAH. In the modeled structure (Fig. 1) the residues Arg<sup>13</sup>, Glu<sup>260</sup>, Glu<sup>353</sup>, and Glu<sup>381</sup> are of particular interest, and to further assess their role we substituted Glu<sup>353</sup>, Glu<sup>381</sup>, and Arg<sup>13</sup> by charged-to-Ala scanning mutagenesis. Substitution of Glu<sup>260</sup>, which (in addition to Arg<sup>13</sup>) participates in the reorientation of the N-terminal tail by repulsion with the phosphate group at Ser<sup>16</sup> (Fig. 1), was also considered. Glu<sup>260</sup> is important in stabilizing the active site structure by forming a salt bridge with Arg<sup>158</sup> (6), and Glu<sup>260</sup> mutations have been found to result in an unfolding and a low catalytic activity of the recombinant.

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<sup>a</sup> M. Thórofsón, unpublished results.
enzymes were preincubated (5 min, 25 °C, pH 7.0) with L-Phe at the same concentrations as in the assay. Vmax (no Phe preincubation) was determined without L-Phe during preincubation but at otherwise identical conditions.

The catalytic activity of the isolated tetrameric forms was measured at pH 7.0 at 25 °C. Vmax (Phe preincubation), the concentration of L-Phe at half maximal activity ([S]0.5), and the Hill coefficient (h) for L-Phe were determined at 75 μM BH4 and variable concentrations of L-Phe (0–5 mM); the enzymes were preincubated (5 min, 25 °C, pH 7.0) with L-Phe at the same concentrations as in the assay. Vmax (no Phe preincubation) was determined without L-Phe during preincubation but at otherwise identical conditions.

| Enzyme | [S]0.5 (L-Phe) | h         | Vmax (Phe preincubation) | Vmax (no Phe preincubation) |
|--------|---------------|-----------|-------------------------|-----------------------------|
|        | μM            |           | nmol Tyr·min⁻¹·mg⁻¹       |                             |
| wt-hPAH| 154 ± 20      | 2.0       | 1100 ± 9                | 3480 ± 165                  |
| Pt-wt-hPAH* | 86 ± 10    | 1.9       | 1510 ± 136              | 3270 ± 85                   |
| S16A   | 160 ± 7       | 2.1       | 900 ± 69                | 3100 ± 12                   |
| S16D   | 117 ± 36      | 2.1       | 1250 ± 69               | 3250 ± 12                   |
| S16E   | 90 ± 36       | 2.1       | 1480 ± 82               | 3250 ± 62                   |
| R13A   | 96 ± 14       | 1.7       | 1261 ± 38               | 3246 ± 83                   |
| E381A  | 105 ± 8       | 2.1       | 1537 ± 57               | 2977 ± 33                   |
| Pt-E381A* | 71 ± 21     | 1.7       | 2019 ± 40               | 3044 ± 83                   |

* Pi, phosphorylated.

According to the modeled structure (Fig. 1, A and C) Arg13 is involved in stabilizing the closed low activity conformation of the nonphosphorylated enzyme by forming salt bridges with Glu353 and Glu381 (see above). As found for the E381A mutant, the form R13A revealed a level of basal activity and an affinity for L-Phe that was higher than for wt-hPAH (Table III). Furthermore, the mutant R13A showed a higher resistance to limited tryptic proteolysis than the nonphosphorylated wild-type form of the enzyme (Fig. 3E), indicating that this residue is an important determinant for the susceptibility of hPAH to limited tryptic proteolysis.

On the other hand, the verification of the role of Arg13 to preserve the correct orientation of the N-terminal tail by establishing electrostatic interactions with the phosphate group or the negative charged residues at position 16 in the phosphorylated form (or in the S16E or S16D mutants) is not a straightforward task. First, the mutant form R13A cannot be phosphorylated because this Arg is part of the specific recognition site of PKA (Arg13-Lys14-Leu15-Ser16) (11). We have prepared the double mutant R13A/S16E to mimic the effect of this mutation in the phosphorylated enzyme. Secondly, the dual role of Arg13 both in maintaining a closed conformation by salt bridging with Glu353 and Glu381 in the nonphosphorylated hPAH (see above) and in keeping the open conformation by interacting with the phosphate group in the phosphorylated enzyme would contribute to a mixed effect of the mutation R13A in the phosphorylated hPAH. In agreement with this assumption, the mutant R13A/S16E in fact revealed kinetic properties intermediate of the enzymes with R13A and S16E.

FIG. 3. Limited tryptic proteolysis of phosphorylated and nonphosphorylated wild-type hPAH and mutant forms at Ser16, SDS-PAGE of the tryptic products (A–D) and quantification of the raw volume of full-length band (molecular mass is ~50 kDa in A and C and 51 kDa in B and D) by densitometric scanning (A280 nm) (E) for nonphosphorylated wt-hPAH (A), phosphorylated wt-hPAH (B), S16A (C), S16E (D), R13A (E), E381A (F), and phosphorylated E381A (G). For A and C the band at about 50 kDa is a multicomponent band. The enzyme samples were incubated for 20 min at 30 °C at different trypsin: protein ratios (μg:μg), i.e. 0:1 (lane 2 in A–D), 0.002:1, 0.005:1, 0.01:1, and 0.1:1 (lane 3 in A–D). Lane 1 in A–D, low molecular mass protein standards, i.e. bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), and carbonic anhydrase (31 kDa).
Phosphorylation and Mutations of Ser\textsuperscript{16} in hPAH

FIG. 4. Effect of phosphorylation and mutations at Ser\textsuperscript{16} on the far UV CD spectra of hPAH. Spectra of nonphosphorylated wt-hPAH (continuous line), phosphorylated wt-hPAH (dotted line), and S16E (dashed line) were taken in 20 mM sodium phosphate and 50 mM KF, pH 7.0, at 25 °C. The concentration of hPAH was 10 μM subunit [Θ], molar ellipticity. The spectrum of the mutant S16A is similar to that of wt-hPAH (continuous line).

the nonphosphorylated and phosphorylated forms (data not shown).

**DISCUSSION**

*In vitro* phosphorylation of mammalian PAH by PKA at the single phosphorylation site Ser\textsuperscript{16} results in an increased basal activity and an apparent increased affinity for the substrate L-Phe (12, 14). Studies on both rPAH and hPAH have revealed an interplay between substrate activation and phosphorylation (12, 15, 16), which is considered to have a physiological relevance (1, 13, 17). Crystallographic analysis of the C-terminally truncated dimeric form of rPAH (residues 1–429) has revealed a similar overall structure of the phosphorylated and nonphosphorylated enzyme including residues 19–427, whereas no electron density was observed for the 18 N-terminal residues (9). This finding supports a rather flexible structure of this N-terminal tail and suggests the possibility that the proposed conformational changes triggered by Ser\textsuperscript{16} phosphorylation (18) are localized to this sequence element. This conclusion is supported by the present modeled structure of residues 1–429 in rPAH, and by site-directed mutagenesis, steady-state enzyme kinetics, CD spectroscopy, and limited tryptic proteolysis (see below). The structures show that phosphorylation triggers a conformational change and reorientation of the N-terminal tail (residues 1–15) (Fig. 1, A and B) because of the formation of a new salt bridge between the phosphate group and the conserved Arg\textsuperscript{13} (Fig. 1, C and D). Thus, the estimated average distance between the N\textsubscript{η} atom of Arg\textsuperscript{13} and the three oxygen atoms of the phosphate group was 3.6 Å. In addition to Arg\textsuperscript{13}, Glu\textsuperscript{280} may also contribute to the reorientation of the N-terminal tail because this residue, located in a loop at the entrance to the active site (Fig. 1), would lead to a movement of the phosphorylated Ser\textsuperscript{16} by electrostatic repulsion. Earlier studies on the effect of substitutions of Ser\textsuperscript{16} in rPAH by negatively charged, positively charged, and uncharged amino acids have revealed that its activation by phosphorylation can be explained by the introduction of a negative charge at this position (40, 41). In the present studies on hPAH, it is shown that the Ser → Glu substitution, and to a lesser extent the Ser → Asp substitution, also results in an increased basal activity (i.e., in the absence of preincubation with L-Phe) and an apparent increased affinity for L-Phe (Table III). Furthermore, these mutant forms are characterized by a decreased susceptibility to limited proteolysis by trypsin, similar to that observed on Ser\textsuperscript{16} phosphorylation (Fig. 3), as well as by a mobility shift on SDS-PAGE (Fig. 2) and an apparent increase of the α-helical content as determined by CD spectroscopy (Fig. 4). Although the introduction of the negatively charged residues (Asp and Glu) mimic Ser\textsuperscript{16} phosphorylation in terms of changes in enzyme kinetics and conformational properties, the effects are quantitatively smaller, notably for the difference in electrophoretic mobility in SDS-PAGE with respect to the nonphosphorylated wild-type form or the S16A mutant. The Ferguson plot analysis of the mobility shift between the SDS-denatured forms of the phosphorylated and nonphosphorylated forms indicates that the two forms (nonphosphorylated and phosphorylated) differ in their apparent Stokes radius, suggesting that the electrostatic interaction (between Arg\textsuperscript{13}/Lys\textsuperscript{14} and the phosphorylated Ser\textsuperscript{16}) results in a local conformational change at the N-terminal tail that is maintained in the partly denatured state. Because the maximum charge of the Glu side chain is −1 compared with −2 for the phosphate group, the electrostatic interaction in the mutant is weaker.

On the basis of the modeled structures (Fig. 1) and mutagenesis at Ser\textsuperscript{16}, Glu\textsuperscript{381}, and Arg\textsuperscript{13}, it is concluded that the positioning of the inhibitory autoregulatory N-terminal sequence in the nonphosphorylated form results in a relatively closed active site crevice structure. On phosphorylation of Ser\textsuperscript{16} a more open active site is obtained, in agreement with the enzyme kinetic data with an increased affinity for the substrate (Table III), which binds close to the iron through interactions with several active site residues including the residues Arg\textsuperscript{279}, Trp\textsuperscript{326}, and Ser\textsuperscript{409} (34). Thus, the modeled structure (Fig. 1, B and D) explains the observed interdependence of L-Phe activation and phosphorylation (12, 14, 18). Moreover, the bent backbone conformation around Ser(P)\textsuperscript{16} may be related to the apparent increase in α-helical content as observed by CD spectroscopy. Interestingly, by Fourier-transform infrared spectroscopy we have previously observed an increase in the apparent α-helical content of the isolated 1–110-residue N-terminal regulatory domain of hPAH and of the iron reconstituted recombinant human tyrosine hydroxylase on phosphorylation of Ser\textsuperscript{16} (23) and Ser\textsuperscript{40} (42), respectively.

Our modeled structure of the full-length enzyme also explains the results obtained by limited proteolysis with trypsin (Fig. 3). Thus, the higher content of noncovalent contacts, mostly van der Waals’s interactions (≤4 Å), between the N-terminal tail and the catalytic domain in the phosphorylated enzyme (Table II) is compatible with a more restrained configuration of the tail (supplemental material) and the decreased susceptibility to limited tryptic proteolysis observed by the phosphorylated wt-hPAH and the S16D and S16E mutant forms (Fig. 3). Furthermore, the R13A mutant also shows a reduced rate of tryptic proteolysis, indicating that this residue is an important determinant for trypsin binding and cleavage of hPAH. Accordingly, the protection against proteolysis observed in the phosphorylated enzyme and in the mutants with acidic residues at the 16 position also seems to be related to the lower accessibility of Arg\textsuperscript{13} to trypsin in these enzymes, in which Arg\textsuperscript{13} forms salt bridges with Asp\textsuperscript{145} and the phosphate group on Ser\textsuperscript{16} (or the carboxylate in the S16D and S16E forms) and thus becomes sandwiched between these residues (Fig. 1D). Arg\textsuperscript{13} is essential for phosphorylation of Ser\textsuperscript{16} (11), as part of the consensus sequence for PKA-catalyzed phosphorylation (Arg(-Asp/Lys)-Xaa-Ser), similar to that found in liver pyruvate kinase (43) and in the α and β regulatory subunits of phosphofructokinase (for review see Ref. 44). Using model heptapeptides corresponding to the phosphorylation site of pyruvate kinase, it was shown that phosphorylation inhibits the rate of
cleavage by trypsin-like enzymes (45), which has been related to an effect of phosphorylation in protein processing and turnover in vivo. Phosphorylation and/or introduction of negatively charged residues at the phosphorylation sites has also been associated with increased protein stability in HPr, a key regulatory protein in bacteria (46), in the ATP2 transcription factor (47), the p27(Kip1) protein (48) and the high mobility group B proteins (49), among others. Although it has been reported that the stabilization induced by phosphorylation of Ser\textsuperscript{16} in the HPr protein is due to an electrostatic interaction between the negatively charged groups and the helix macrodipole (46, 50), there are no direct insights into the specific mechanisms at the atomic level leading to this increase in stability for the other proteins. The structural model for PAH in Fig. 1 (B and D) is also in agreement with the finding that the phosphorylated form is better mimicked by the S16E mutant form than by S16D (Ref. 41 and this work). Indeed, a stronger interaction is observed between the Arg\textsuperscript{13} and the longer glutamate side chain than with the aspartate (data not shown).

Thus, the results from this study give further credit to the proposal that phosphorylation of PAH induces a local conformational change at the N-terminal autoregulatory sequence, brought about mainly by an electrostatic interaction between the phosphate group and Arg\textsuperscript{13}. The conformation of the phosphorylated enzyme results in facilitated access of the substrate to the active site.

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Phosphorylation and Mutations of Ser<sup>16</sup> in Human Phenylalanine Hydroxylase: KINETIC AND STRUCTURAL EFFECTS

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