Deamidations in Recombinant Human Phenylalanine Hydroxylase
IDENTIFICATION OF LABILE ASPARAGINE RESIDUES AND FUNCTIONAL CHARACTERIZATION OF ASN \rightarrow ASP MUTANT FORMS*  

Recombinant human phenylalanine hydroxylase (hPAH) expressed in Escherichia coli for 24 h at 28 °C has been found by twodimensional electrophoresis to exist as a mixture of four to five molecular forms as a result of nonenzymatic deamidation of labile Asn residues. The multiple deamidations alter the functional properties of the enzyme including its affinity for L-phenylalanine and tetrahydrobiopterin, catalytic efficiency, and substrate inhibition and also result in enzyme forms more susceptible to limited trypic proteolysis. Asn32 in the regulatory domain deamidates very rapidly because of its nearest neighbor amino acid amidated 24-h expressed wt-hPAH. Moreover, deamidation in proteins and peptides has been shown to be dependant on pH, temperature, and ionic strength (7) and on intrinsic factors like the nearest neighbor amino acids, particularly the Asp substituion in a variable ratio, sometimes with iso-Asp as the main product (5, 6). The rate of Asn deamidation in proteins and peptides has been shown to be dependant on pH, temperature, and ionic strength (7) and on intrinsic factors like the nearest neighbor amino acids, particularly the residue in the (n + 1) position (8–10). The deamidation proceeds via a cyclic succinimide intermediate (6), and with glycine in the n + 1 position the rate of deamidation is unusually rapid, whereas all the other 19 amino acid residues are more sterically hindered in the formation of the cyclic intermediate (11). Moreover, the rate of deamidation is also determined by the protein secondary and tertiary structure because the necessary flexibility for the formation of the cyclic intermediate may be limited in proteins (12). The fact that deamidation is an

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Phenylalanine hydroxylase (PAH, 1 phenylalanine 4-monooxygenase, EC 1.14.16.1) is a non-heme iron monooxygenase that catalyzes the hydroxylation of L-phenylalanine (L-Phe) to L-tyrosine (L-Tyr) in the presence of the natural cofactor (6R)-1-erythro-5,6,7,8-tetrahydrobiopterin (H4biopterin) and dioxygen. Mutations in the human enzyme (hPAH) leading to altered kinetic properties or reduced stability of the enzyme are associated with the autosomal recessive disorder phenylketonuria (PKU). hPAH isolated from liver (2) and as recombinant enzyme expressed in Escherichia coli (3, 4) has been found to exist as a mixture of 4–5 molecular forms with the same apparent subunit molecular mass, but with different isoelectric points (pI). Isoelectric focusing and two-dimensional electrophoresis of recombinant hPAH expressed in E. coli for 24 h at 28 °C revealed five components of decreasing staining intensity and decreasing pI (denoted hPAH I-V). This microheterogeneity was shown to be the result of nonenzymatic deamidations of Asn residues (1, 4). Mainly one band with the highest pI (hPAH I) was, however, detected after a short induction period of 2 h at 28 °C, and this form was considered to represent the newly synthesized and most native, nondeamidated form of the enzyme. Thus, the microheterogeneity pattern is highly dependent on the induction time with IPTG in E. coli (4). Due to the relatively high rate of deamidation, the labile amide-containing residues have been considered to be Asn residues (4), and this conclusion has recently been confirmed by the demonstration of iso-Asp in several tryptic peptides of the highly deamidated full-length wt-hPAH (1). In the deamidation reaction Asn is converted to Asp and iso-Asp in a variable ratio, sometimes with iso-Asp as the main product (5, 6). The rate of Asn deamidation in proteins and peptides has been shown to be dependant on pH, temperature, and ionic strength (7) and on intrinsic factors like the nearest neighbor amino acids, particularly the residue in the (n + 1) position (8–10). The deamidation proceeds via a cyclic succinimide intermediate (6), and with glycine in the n + 1 position the rate of deamidation is unusually rapid, whereas all the other 19 amino acid residues are more sterically hindered in the formation of the cyclic intermediate (11). Moreover, the rate of deamidation is also determined by the protein secondary and tertiary structure because the necessary flexibility for the formation of the cyclic intermediate may be limited in proteins (12). The fact that deamidation is an

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ordered process adds to the complexity, as structural changes induced by deamidation of one residue may influence subsequent sequential deamidation reactions (reviewed in Ref. 13). The rate of deamidation in model peptides has also been shown to be inversely proportional to the extent of $\alpha$-helicity which suggests that Asn deamidation occurs preferentially in nonhelical structural elements (14). The deamidation of Asn introduces an additional negative charge (Asp or iso-Asp) that may result in significant conformational changes of the protein as well as altered functional properties. A difference in functional properties was first described for the multiple deamidated forms of bovine heart cytochrome $c$, i.e., a deactivation (15), and more recently also shown to be the case for wt-hPAH, but in this case with an activation of the enzyme (4). A comparison of the catalytic properties of nondeamidated and highly deamidated mutant forms with special reference to its effect on the rate of phosphorylation of Ser$^{16}$ by PKA, used as a conformational probe.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—The mutations in the catalytic domain were introduced into the wt-hPAH cDNA by PCR-based site-directed mutagenesis (18) using the pMAL-hPAH vector, containing the factor Xa cleavage site (New England Biolabs) as a template (19), and the specific oligonucleotide primers listed in Table I. The target sequences for mutagenesis were the restriction endonuclease fragments XhoI/BamHI (N133D, N167D, and N167I), BamHI/II (N376D, N393D, N401D, and N426D). The three mutations in the regulatory domain and the double mutant N32D/N376D were introduced into the pMAL-hPAH expression system containing the enterokinase cleavage site (D4K) (New England Biolabs) using the QuickChange™ site-directed mutagenesis kit (Stratagene) and the specific oligonucleotides listed in Table I. Authenticity of mutagenesis was verified by DNA sequencing using the malE and 13B oligonucleotides (20) and the Big Dye™ Terminator Ready Reaction Mix (PerkinElmer Life Sciences) in an ABI Prism™ 377 DNA Sequencer (PerkinElmer Life Sciences). MWG-Biotech AG provided primers for mutagenesis and sequencing. Some of the mutants were also verified by MALDI-TOF mass spectrometry of the isolated recombinant enzyme (see "Results").

Expression and Purification of the Enzymes—The wild-type and the mutant forms of hPAH were expressed as fusion proteins in E. coli (4). On the basis of a computer algorithm that estimates the deamidation rates by taking into account the nearest neighbor amino acids and the three-dimensional structure of the protein (16), the relative deamidation rates of all the Asn residues in hPAH have been predicted (17). We have recently verified that Asn$^{133}$ (followed by a glycine residue), Asn$^{30}$, and Asn$^{30}$ in a loop region of the N-terminal autoregulatory sequence (residues 19–33) of wt-hPAH are among the susceptible residues (1). On the basis of the computational method two other labile residues have been predicted to be located in the catalytic domain structure, i.e., Asn$^{133}$ and Asn$^{376}$ (17). Here we present experimental data verifying this prediction based on MALDI-TOF mass spectrometry of tryptic peptides and site-directed mutagenesis. Mutagenesis was also used to characterize the structural and functional effects of single Asn $\rightarrow$ Asp substitutions at 8 alternative positions in the catalytic domain. Moreover, the functional effects of deamidation of Asn$^{32}$ are further studied in mutant forms with special reference to its effect on the rate of phosphorylation of Ser$^{16}$ by PKA, used as a conformational probe.
isotropically deconvolute the mass spectra (Sierra Analytics, Modesto, CA). The deconvolution method was particularly useful to detect labile Asn residues in a peptide because deamidation of Asn to Asp/iso-Asp increases the monoisotopic mass ([M + H]+) of the peptide by only 1 Da.

**Assay of hPAH Activity**—The hPAH activity was assayed as described (21), but the catalase concentration was 0.1 µg/µl, and the enzyme was activated by prior incubation (5 min) with t-Phe. Moreover, 0.5% (v/v) bovine serum albumin was included in the reaction mixture to stabilize the diluted purified enzyme. The enzyme source was either oligomerization (tetramers > dimers) similar to that of wt-hPAH (21, 26, 27). Moreover, after their cleavage by restriction protease, no significant amount of aggregated enzyme forms was observed in the size-exclusion chromatographic profiles. One-dimensional SDS-PAGE analysis of all the mutant forms revealed the same relative electrohoretic mobility as for the wt-hPAH protoner, both as uncleaved and cleaved fusion protein (data not shown).

**Mass Spectrometry**—To identify the labile Asn residues in wt-hPAH and hPAH with the N167I mutant, and the reaction time was 1 min. The steady-state kinetic data were analyzed by non-linear regression analysis using the SigmaPlot® Technical Graphing Software and the modified Hill equation of LiCata and Allewell (22) for cooperative substrate binding as well as substrate inhibition (4, 23). In some experiments 1 mM t-Phe was added either at the start of the preincubation period or together with 75 µM pterin cofactor (B2pterin) at the initiation of the hydroxylation reaction. A 3-min time course was then followed in order to study the effect of preincubation with t-Phe on the specific activity of the wild-type and mutant forms of hPAH.

**Steady-state Kinetic Properties of Tetrameric Wild-type and Its Mutant Forms**—The introduction of extra negative charges in wt-hPAH as a result of nonenzymatic deamidation during a 24-h expression period at 28 °C in the E. coli pMal system has been shown to increase the solubility and recovery of recombinant wt-hPAH relative to the 2-h expressed enzyme (4). The expression of the Asn → Asp mutant forms of hPAH resulted in a variable but generally increased yield of fusion proteins (relative to wt-hPAH) after an IPTG induction period of only 2 h at 28 °C. Size-exclusion chromatography of the affinity-purified mutant fusion proteins (data not shown) revealed a pattern of oligomerization (tetramers > dimers) similar to that of wt-hPAH (21, 26, 27). Moreover, after their cleavage by restriction protease, no significant amount of aggregated enzyme forms was observed in the size-exclusion chromatographic profiles. One-dimensional SDS-PAGE analysis of all the mutant forms revealed the same relative electrohoretic mobility as for the wt-hPAH protoner, both as uncleaved and cleaved fusion protein (data not shown).

**Expression and Purification of wt-hPAH and Its Mutant Forms**—The introduction of extra negative charges in wt-hPAH as a result of nonenzymatic deamidation during a 24-h expression period at 28 °C in the E. coli pMal system has been shown to increase the solubility and recovery of recombinant wt-hPAH relative to the 2-h expressed enzyme (4). The expression of the Asn → Asp mutant forms of hPAH resulted in a variable but generally increased yield of fusion proteins (relative to wt-hPAH) after an IPTG induction period of only 2 h at 28 °C. Size-exclusion chromatography of the affinity-purified mutant fusion proteins (data not shown) revealed a pattern of oligomerization (tetramers > dimers) similar to that of wt-hPAH (21, 26, 27). Moreover, after their cleavage by restriction protease, no significant amount of aggregated enzyme forms was observed in the size-exclusion chromatographic profiles. One-dimensional SDS-PAGE analysis of all the mutant forms revealed the same relative electrohoretic mobility as for the wt-hPAH protoner, both as uncleaved and cleaved fusion protein (data not shown).

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**Expression and Purification of wt-hPAH and Its Mutant Forms**—The introduction of extra negative charges in wt-hPAH as a result of nonenzymatic deamidation during a 24-h expression period at 28 °C in the E. coli pMal system has been shown to increase the solubility and recovery of recombinant wt-hPAH relative to the 2-h expressed enzyme (4). The expression of the Asn → Asp mutant forms of hPAH resulted in a variable but generally increased yield of fusion proteins (relative to wt-hPAH) after an IPTG induction period of only 2 h at 28 °C. Size-exclusion chromatography of the affinity-purified mutant fusion proteins (data not shown) revealed a pattern of oligomerization (tetramers > dimers) similar to that of wt-hPAH (21, 26, 27). Moreover, after their cleavage by restriction protease, no significant amount of aggregated enzyme forms was observed in the size-exclusion chromatographic profiles. One-dimensional SDS-PAGE analysis of all the mutant forms revealed the same relative electrohoretic mobility as for the wt-hPAH protoner, both as uncleaved and cleaved fusion protein (data not shown).
Labile Asp Residues in Human Phenylalanine Hydroxylase

15145

Fig. 1. MALDI-TOF mass spectra of the tryptic peptides Thr<sup>372</sup>-Lys<sup>396</sup> and Phe<sup>131</sup>-Lys<sup>396</sup> of wt-hPAH containing labile Asn residues. The mass spectra were isotopically deconvoluted, and only the monoisotopic mass peaks ([M + H]<sup>+</sup>) are shown. A, the peptide Thr<sup>372</sup>-Lys<sup>396</sup> from wt-hPAH expressed for 24 h at 28 °C; B, the same peptide from the Asn<sup>376</sup>→Asp mutant form; C, the peptide Phe<sup>131</sup>-Lys<sup>396</sup> from ΔN-(1–102)/AC(429–452)-hPAH expressed for 24 h at 28 °C; D, the same peptide from the Asn<sup>376</sup>→Asp mutant form.

An increased positive kinetic cooperativity of L-Phe binding as well as a substrate inhibition are also characteristic properties of multiple deamidated (24-h induction at 28 °C) wt-hPAH (4). The N32D, N133D, N376D, N401D, and N32D/N376D mutant tetramers revealed all the same positive cooperativity (1.8 < h < 2.0) as the multiple deamidated wt-hPAH as well as a substrate inhibition at L-Phe concentrations >1 mM (Table III). By contrast, the other Asn→Asp mutant forms did not reveal any substrate inhibition within the selected concentration range of L-Phe (≤4 mM). For the N133D and N426D mutant forms a positive cooperativity (1.4 < h < 1.6) comparable with wt-hPAH (2-h induction) (h = 1.5) was observed, whereas no significant cooperativity (0.8 < h < 1.2) was found for the N207D, N223D, and N393D mutant tetramers.

A slightly reduced affinity for the natural pterin cofactor has been observed for multiple deamidated hPAH when compared with the nondeamidated enzyme (4), and in the present study a 29% increase in the K<sub>m</sub> (H<sub>4</sub>biopterin) value was observed for the wt-hPAH (24-h induction). The N167D, N223D, N376D, and N401D mutant forms also demonstrated a significant increase in the K<sub>m</sub> value for the natural pterin cofactor, i.e. by 19–39%, compared with wt-hPAH (2-h induction) (Table III). By contrast, the N133D and N426D mutants revealed an affinity for the pterin cofactor comparable with wt-hPAH (2-h induction), whereas a reduction in the K<sub>m</sub> value by 74 and 35% was observed for the N207D and N393D mutants, respectively (Table III).

Effect of Preincubation with L-Phe on the Catalytic Activity—A characteristic property of tetrameric wt-hPAH is also that preincubation with L-Phe resulted in an activation of the enzyme catalytic activity, and that the measured fold activation varied with the time of preincubation (4) due to a relatively slow substrate-induced hysteretic conformational transition (29). To study the effect of the Asn→Asp mutations on the activation process, the catalytic activities of both nondeamidated wt-hPAH and the mutant tetramers (2-h induction) were measured in the presence and absence of preincubation with 1 mM L-Phe. Table III shows that a 5–7-fold activation was observed for the N133D, N376D, N401D, and N426D mutant tetramers, which is in the same range of activation as observed for the 24-h-induced multiple deamidated wt-hPAH (5.8-fold) (Table III). The N167D, N223D, and N393D mutant tetramers revealed a lower degree of activation (i.e. 2.7–3.8-fold) than the wt-hPAH (2-h induction) (4.9-fold), whereas no L-Phe activa-
tion was observed for the N207D mutant form (Table III). Interestingly, the Asn32 → Asp mutant tetramer revealed the same degree of activation as the wt-hPAH (2-h induction), whereas an increased basal catalytic activity with almost no further stimulation by preincubation with Phe was observed for the Gly33 → Ala/Val mutations (Table III).

Limited Proteolysis by Trypsin—Limited proteolysis by trypsin has been shown to represent a sensitive conformational probe in wt-hPAH (4) and some of its disease-associated mutant forms (26). That nondeamidated hPAH (2-h induction) was more resistant to limited proteolysis by trypsin than multiple deamidated hPAH (24-h induction) (4) was confirmed in the present time course studies, with about 85 and 52% of the full-length protomer recovered for the nondeamidated and multiple deamidated forms of wt-hPAH, respectively, after incubation for 15 min at a trypsin:protein ratio of 1:200 (by mass) (data not shown). Moreover, 6 of 8 Asn → Asp mutant tetramers (i.e. N167D, N207D, N223D, N376D, N393D, and N401D) revealed a variable but increased susceptibility to limited proteolysis when compared with the nondeamidated wild-type enzyme (2-h induction). The N207D mutant form was most susceptible to degradation, because only 19% full-length protomer

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**Fig. 2.** The effect of l-Phe concentration on the catalytic activity of the double mutant form N32D/N376D-hPAH. For assay conditions of the isolated tetrameric enzyme preparation and nonlinear regression analysis, see “Experimental Procedures.” A, N32D/N376D-hPAH; B, wt-hPAH (2-h induction); C, wt-hPAH (24-h induction). In all graphs the experimental (●) and fitted (○) data are shown. The kinetic constants are summarized in Table III.

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Phosphorylation with PKA and tryptic proteolysis, the 32P-labelling at pH 7 and 37°C sensitized the enzyme toward activation by L-Phe (30). More-over, IPTG (2-h induction) (data not shown).

Phosphorylation of hPAH—In wt-hPAH Ser16 was a substrate for the catalytic subunit of PKA, and its phosphorylation sensitized the enzyme toward activation by L-Phe (30). Moreover, the rate of this phosphorylation was conformation-sensitive and was stimulated by L-Phe binding, whereas BH4 acted as a negative effector (30). In this study, we have further observed that the initial rate of phosphate incorporation was about 20% higher (p < 0.003) for the multiple deamidated enzyme (24-h induction) than for the nondeamidated enzyme (2-h induction) hPAH (Fig. 3). The N32D mutant form (2-h induction) also resulted in an enhanced rate of phosphorylation by PKA (Fig. 3), comparable (p < 0.2) to the deamidated wt-hPAH (24-h induction), whereas the G33A/G33V mutant forms (2-h induction) and the G33A (24-h induction) were phosphorylated with a similar initial rate as the nondeamidated wt (2-h expression) enzyme (Fig. 3).

Reversed-phase Chromatography of Tryptic Peptides—Together with Asn28 and Asn30, the very labile Asn32 is part of a cluster of Asn residues in the 28-residue N-terminal tryptic peptide (Leu15-Lys42), which also includes Ser16. Upon its phosphorylation with PKA and tryptic peptides, the 32P-labeled peptides were resolved by reversed-phase chromatography into some minor phosphopeptides and a major component (tR ~34 min), which revealed a time-dependent shift to a more hydrophilic position (tR ~31 min) when incubated at 37 °C (1). When the phosphopeptides resulting from tryptic proteolysis of the N32D and G33A/G33V mutant enzymes were subjected to reversed-phase chromatography, the major peptide of N32D revealed the expected retention time of tR ~31 min, while that of G33A/G33V mutant derived peptide eluted with a tR ~36 min, and none of them shifted on further incubation at pH 7 and 37 °C (Fig. 4) (results not shown for G33V). The minor components in the chromatograms can be explained as the result of alternative tryptic cleavage sites (1).

**DISCUSSION**

The microheterogeneity of recombinant wt-hPAH expressed in E. coli observed on isoelectric focusing and two-dimensional electrophoresis has been shown to be the result of multiple nonenzymatic deamidations of Asn residues (1, 4). In general, such protein deamidations have been related to their aging and turnover in vivo (10, 31), which is also the case for hPAH. First, on expression of recombinant hPAH in E. coli, this post-translational modification was found to increase progressively with increasing induction time with IPTG and not to be related to the purification or storage of the enzyme. Second, highly de-amidated forms of the enzyme are more susceptible to limited proteolysis by trypsin as compared with the nondeamidated form (4). Finally, PAH purified from human and monkey liver has also been reported to consist of multiple molecular forms on two-dimensional electrophoresis (2), which suggests that non-enzymatic deamination of Asn residues plays a role in the regulation of the catalytic activity and the cellular turnover of PAH in the hepatocytes in vivo.

**Asn Residues Undergoing Deamidation in Recombinant hPAH**—Based on a recently developed computer algorithm (10, 16), several candidate Asn residues have been predicted in the regulatory and catalytic domains of hPAH (17). The deamidation of the predicted residues in the autoregulatory sequence of the N-terminal regulatory domain (Asn3, Asn10, and Asn17) has been confirmed recently (1) by MALDI-TOF mass spectrometry, with Asn10 as the most labile residue. This deamidation has been studied further in the present study by reversed-phase chromatography of the phosphorylated (32P-labeled) N-terminal tryptic peptide (Leu15-Lys42) of the mutant N32D and G33A/G33V enzymes. The phosphopeptide of the N32D mutant form was eluted with the same retention time as the deamidated peptide of wt-hPAH incubated at 37 °C (main peak at tR ~31 min) (Fig. 4) (1). By contrast, the phosphopeptides of the G33A/G33V mutant enzymes were eluted with a higher retention time (tR ~36 min) (Fig. 4) comparable with that observed for the peptide of nondeamidated wt-hPAH (main peak at tR ~34 min) (1); the small difference in retention time may be explained by the substitution of a Gly with a larger aliphatic residue. Thus, as expected from studies on model peptides (10), the Gly28→Ala/Val mutations stabilize Asn28 (see also Fig. 3).

**On the basis of the amino acid sequence and higher order structure, the mentioned computer algorithm (16, 32) estimated a very low deamidation coefficient (Cp < 5.5) for Asn28,
indicating that it is one of the most labile Asn residues in the catalytic domain (Table IV). This prediction was verified by MALDI-TOF mass spectrometry and site-directed mutagenesis. Thus, the tryptic peptide Thr373–Lys392 of the wt-hPAH (24-h induction) was resolved into two monoisotopic ([M/H]+11001) peaks corresponding to nondeamidated (Asn) forms (Table II) and deamidated (Asp/iso-Asp) forms (Fig. 1A), whereas the N376D mutant revealed only one monoisotopic peak corresponding to an Asp residue in this 25-residue peptide (Fig. 1B). By contrast, two alternative deamidation coefficients were estimated for Asn133 on the basis of the three-dimensional structure of the ΔN-(1–117)-hPAH (CD = 4.1) and the ΔN-(1–102)/ΔC-(429–452)-hPAH (CD = 73) truncated forms (Table IV). The different CD values probably reflect an effect of the regulatory and tetramerization domains on the stability of Asn 133. In the full-length wt-hPAH the native three-dimensional structure may contribute to additional structural constraints that protect against deamidation of Asn133. Thus, only one monoisotopic peak, corresponding to an asparagine residue at position 133, was found in the Phe131–Lys150 tryptic peptide for the wt-hPAH (24 h), and only a minor deamidated form was recovered in the same peptide from the ΔN-(1–102)/ΔC-(429–452)-hPAH truncated from (Fig. 1C). These results, together with the deamidation coefficient estimated for this residue in the phosphorylated form of rPAH-ΔC22 (17), show that Asn133 represents the second labile Asn in the catalytic domain of hPAH.

Site-directed Mutagenesis—Mutagenesis (Asn → Asp) of the three labile Asn residues Asn32, Asn376, and Asn133 has provided useful models to study the contribution of each single Asn deamidation to the changed functional properties that occur in wt-hPAH on long term expression in E. coli. It should be noted, however, that the introduction of a negative charge by Asn → Asp mutagenesis does not fully account for the effect of deamidation of wt-hPAH in which iso-Asp might also be formed. The generation of an iso-Asp residue, which is a regular product of the deamidation reaction, also results in a conformational change because it adds an extra carbon to the polypeptide backbone (33). Thus, the formation of iso-Asp has been shown recently for the deamidation of alternative Asn residues in

![Fig. 3. The effect of deamidation of Asn32 on the initial rate of phosphorylation of hPAH at Ser16 by PKA c-subunit.](http://www.jbc.org/)

A, time course of phosphate incorporation of the deamidated wt-hPAH (24-h induction). The data points correspond to the average experimental values for the incorporation of 32P (n = 3). The initial rate of phosphorylation was calculated by the 15 individual data points obtained within the first 2.5 min, and the initial rate v0 (tangent to the curve at zero time) was calculated by nonlinear regression analysis. B, the bars with the mean ± S.E. represent the initial rates for the phosphorylation of deamidated and nondeamidated hPAH. Analysis of variance of the initial rate means indicated a statistical significant difference between the wt-hPAH (24 h) versus wt-hPAH (2 h) (p < 0.003) and the N32D (2 h) versus wt-hPAH (2 h) (p < 0.005). There was no statistical significant difference between the wt-hPAH (2 h) versus G33A (2 h), G33V (2 h), or G33A (24 h) (p < 0.6) nor between the wt-hPAH (24 h) versus N32D (2 h) (p < 0.2).
wt-hPAH including Asn$^{32}$ (1), as expected from the Asn$^{32}$–Gly$^{33}$→Ala mutant forms of hPAH. The full-length mutant tetramers were phosphorylated at Ser$^{16}$ by PKA and digested with trypsin. The phosphopeptides were subjected to reversed-phase chromatography at time 0 (→) and after 32 h of incubation (−−−) in the phosphorylation medium at pH 7 and 37 °C. 250-μl fractions were collected every 15 s, followed by scintillation counting and analysis of the data by the PeakFit software program (SPSS Inc., Chicago). The elution pattern of the phosphopeptides revealed a main peak at $t_r \sim 31$ min for the N32D and at $t_r \sim 36$ min for the G33A mutant form. The heterogeneity of the eluted phosphopeptides may be related to alternative cleavage sites for tryptic proteolysis (1).

Fig. 4. Reversed-phase chromatography of $^{32}$P-labeled tryptic phosphopeptides of Asn$^{32}$→Asp and Gly$^{33}$→Ala mutant forms of hPAH. The full-length mutant tetramers were phosphorylated at Ser$^{16}$ by PKA and digested with trypsin. The phosphopeptides were subjected to reversed-phase chromatography at time 0 (→) and after 32 h of incubation (−−−) in the phosphorylation medium at pH 7 and 37 °C. 250-μl fractions were collected every 15 s, followed by scintillation counting and analysis of the data by the PeakFit software program (SPSS Inc., Chicago). The elution pattern of the phosphopeptides revealed a main peak at $t_r \sim 31$ min for the N32D and at $t_r \sim 36$ min for the G33A mutant form. The heterogeneity of the eluted phosphopeptides may be related to alternative cleavage sites for tryptic proteolysis (1).

The N32D mutation revealed catalytic properties similar to that of multiple deamidated wt-hPAH (24 h expression), i.e. an increased catalytic efficiency and an increased positive cooperativity of L-Phe binding and substrate inhibition (Table III), whereas substitution of Gly$^{33}$ with Ala/Val residues, which prevents the deamidation of Asn$^{32}$, resulted in enzyme preparations with kinetic properties similar to that observed for wt-hPAH (2-h expression). Moreover, the N32D mutant form and the wt-hPAH (24 h) showed similar increased rates of phosphorylation of Ser$^{16}$ by PKA when compared with the G33A and G33V mutant forms and the wt-hPAH (2 h) (Fig. 3). Deamidation of Asn$^{32}$ into an Asp/iso-Asp residue adds a negative charge to the side chain that results in an electrostatic repulsion notably from Asp$^{34}$ (1) and results in a conformational change, as determined by its effect on the rate of phosphorylation of Ser$^{16}$ by PKA. Deamidation of Asn$^{32}$ seems to be the only determinant for this effect, because the partial deamidation of the other asparagine residues, which occur in the G33A mutant form on 24-h induction, did not affect the initial rate of phosphorylation (Fig. 3).

In the catalytic domain, Asn$^{376}$ was shown to represent the most labile Asn residue. In the crystal structure it is located at the beginning of a loop (designated the 380-loop) that represents one limiting side of the active site crevice structure (35) (Table IV; Fig. 5). On binding of the pterin cofactor (H$_4$bipterin) at the active site a conformational change, including a movement of this loop, has been observed for a double truncated form (“catalytic domain”) of hPAH (35). On this background, it was not unexpected to find that the steady-state kinetic parameters of the N376D mutant tetramer were comparable with those observed for multiple but partially deamidated wt-hPAH (24-h induction) (Table III). Its $V_{\text{max}}$ was slightly higher than that of the multiple deamidated wt-hPAH and, in addition, a reduction by 33% in the $[S]_{0.5}$ (L-Phe) value and an increase (by 39%) in the $K_m$ (H$_4$bipterin) was observed as compared with 46 and 29%, respectively, for the multiple deamidated (24-h induction) wt-hPAH (Table III). The N376D mutant form also revealed an increase in the fold activation on preincubation with L-Phe when compared with the wt-hPAH (2-h induction) (Table III), similar to that observed for the multiple deamidated hPAH (24-h induction). It has been proposed previously (34) that the autoregulatory sequence of the N-terminal regulatory domain partly blocks the access of substrate to the active site in the catalytic domain. On preincubation with L-Phe, there is a global conformational change in the enzyme (29), including a possible repositioning of the N-terminal “arm” relative to the active site (34), and an activation of the enzyme. A hinge region centered at Gly$^{33}$ has been proposed to be involved in this repositioning (34), on the basis of a high crystallographic B-factor in this region of the rat enzyme, compatible with a high mobility. Although a movement at this
hinge region is probably involved in the regulation of the access of substrate to the active site, deamidation of Asn\textsuperscript{32} does not seem to be involved in the increased L-Phe induced activation observed for the deamidated wt-hPAH (24 h), because the N32D mutant form revealed a similar degree of substrate activation as the wt-hPAH (2 h) (Table III). By contrast, in the Gly\textsuperscript{33}Ala/Val mutant forms the orientation of this arm seems to generate a more open entrance for the substrate to the active site, and thus explains their higher basal catalytic activity and reduced activation by L-Phe preincubation. In the catalytic domain, deamidation of Asn\textsuperscript{376} significantly contributes to the increased substrate activation, as seen from the data (Table III) obtained for the N376D and the N32D/N376D mutant forms, when compared with the wt-hPAH (2 h). The second labile Asn residue in the catalytic domain, Asn\textsuperscript{133}, is located in a long (18 residue) loop structure between the C\textsubscript{1/}H\textsubscript{9251}\textsuperscript{1}- and C\textsubscript{1/}H\textsubscript{9251}\textsubscript{2a}-helix (Table IV; Fig. 5), and a minor partial deamidation of Asn 133 was observed in enzyme preparations recovered after 24-h expression. However, the N133D mutant tetramer revealed steady-state kinetic properties comparable with that observed for the nondeamidated wt-hPAH (2 h) (Table III). These results, together with the mass spectrometry data, indicate that deamidation of Asn\textsuperscript{133} is unlikely to contribute to the kinetic

### Table IV

An overview of the nearest neighbor amino acids, secondary structure, and three-dimensional structural interactions of the Asn residues in the catalytic domain of hPAH and their predicted deamidation rates

<table>
<thead>
<tr>
<th>Asn residue</th>
<th>Neighbor residues</th>
<th>$t^{1/2}$ mg (days)</th>
<th>$t^{1/2}$ mg (days)</th>
<th>Secondary structure</th>
<th>Three-dimensional localization</th>
<th>$CD$ coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>133</td>
<td>Phe Gly</td>
<td>60.0</td>
<td>Between C\textsubscript{1}/helix and C\textsubscript{2a}/helix</td>
<td>None</td>
<td>On the surface</td>
<td>4.173*</td>
</tr>
<tr>
<td>167</td>
<td>Ala Tyr</td>
<td>70.6</td>
<td>Just after C\textsubscript{2b}/helix</td>
<td>Asn\textsuperscript{197} N82-Asp\textsuperscript{163} carbonyl oxygen (3.10 Å)</td>
<td>On the surface</td>
<td>740</td>
</tr>
<tr>
<td>207</td>
<td>Glu His</td>
<td>10.2</td>
<td>In the middle of C\textsubscript{4}/helix</td>
<td>Asn\textsuperscript{207} N82-Ala\textsuperscript{202} carbonyl oxygen (3.13 Å)</td>
<td>On the surface</td>
<td>85</td>
</tr>
<tr>
<td>223</td>
<td>Glu Ile</td>
<td>298</td>
<td>In the loop between C\textsubscript{4}/ and C\textsubscript{5}/helix</td>
<td>Asn\textsuperscript{223} N82-Gly\textsuperscript{218} carbonyl oxygen (3.02 Å) Asn\textsuperscript{223} O81-H\textsubscript{2}O-Ile\textsuperscript{224} carbonyl oxygen (2.89 and 2.72 Å)</td>
<td>On the surface (kept in position by H-bonding)</td>
<td>40</td>
</tr>
<tr>
<td>376</td>
<td>Ile Tyr</td>
<td>94.5</td>
<td>Between C\textsubscript{11}/ and C\textsubscript{12}/helix</td>
<td>None</td>
<td>On the surface, in the 380 loop that covers the active site crevice</td>
<td>5.5</td>
</tr>
<tr>
<td>393</td>
<td>Ser Asp</td>
<td>27.4</td>
<td>Just in front of C\textsubscript{12}/helix</td>
<td>Asn\textsuperscript{393} N82 and O81-Ser\textsuperscript{298} O\textsubscript{7} (2.96 and 2.95 Å)</td>
<td>On the surface</td>
<td>230</td>
</tr>
<tr>
<td>401</td>
<td>Val Phe</td>
<td>68.3</td>
<td>In the middle of C\textsubscript{12}/helix</td>
<td>None</td>
<td>On the surface</td>
<td>350</td>
</tr>
<tr>
<td>426</td>
<td>Leu Thr</td>
<td>52.4</td>
<td>Just before C\textsubscript{14}/helix (the beginning of the tetramerization motif)</td>
<td>Asn\textsuperscript{426} O81-Phe\textsuperscript{410} main chain N (2.71 Å)</td>
<td>Solvent-exposed</td>
<td>2100</td>
</tr>
</tbody>
</table>

*Deamidation coefficient predicted from the three-dimensional structure of the ΔN(1–102)/ΔC(428–452)-hPAH (Protein Data Bank code 1PAH) (17).

![Figure 5. Interactions of the asparagine residues in the catalytic domain with the neighbor amino acids in the hPAH structure.](image-url)
Labile Asp Residues in Human Phenylalanine Hydroxylase

15151

differences observed between the 2- and the 24-h expressed enzyme.

Considering all the data discussed above, the double mutant form N32D/N376D represents so far the best model enzyme which mimics the highly deamidated wt-hPAH (24 h). It is also a protein preparation with less microheterogeneity and may thus represent an interesting molecular form in the current efforts to crystallize and solve the structure of the full-length tetrameric enzyme. Thus, nonenzymatic deamidation of Asn residues introduces a time-dependent microheterogeneity that may be related to the reported time-dependent loss of diffraction power of the hPAH crystals (36).

Structural and Functional Considerations of the Stable Asn Residues in the Catalytic Domain—Although the six stable Asn residues observed in the catalytic domain of hPAH are all located on the surface (Table IV), their nearest neighbor amino acids and the structural constraints in the polypeptide backbone represents the protection against their deamidation (17) (Table IV). Asn167, Asn207, Asn393, Asn401, and Asn426 have rather long predicted half-times for deamidation (between 10 and 70 days) based on their nearest neighbor amino acids (10, 17), rather long predicted half-times for deamidation (between 10 and 70 days) based on their nearest neighbor amino acids (10, 17), and they are in addition stabilized by hydrogen bonds and/or by their location in α-helical structures (Table IV), which results in high deamidation coefficients (40 < C_D < 2100) (Table IV) (17). This computational prediction of stable Asn residues was confirmed by MALDI-TOF mass spectrometry of the corresponding tryptic peptides from the 24-h expressed wt-hPAH with single monoisotopic mass peaks, corresponding to fully amidated tryptic peptides as seen for the 2-h expressed enzyme (Table II). Moreover, five residues (Asn167, Asn207, Asn223, Asn393, and Asn426) are involved in at least one stabilizing intrasubunit hydrogen bond (Fig. 5). On Asn → Asp mutation of these residues, their intrasubunit and solvent contacts change more than for the nonhydrogen-bonded residues (Asn133, Asn376, and Asn401).

Asn167 is located just after the Co2b-helix and is stabilized by a single hydrogen bond to the carbonyl oxygen of Asp163 (Table IV; Fig. 5). Interestingly, in the N167D mutant tetramer the repulsive electrostatic interaction between the negatively charged carboxyl groups of Asp167 and Asp163 resulted in similar kinetic properties of the mutant form as the wt-hPAH (24-h induction) (Table III). The importance of the conserved Asn residue at this position is further supported by the finding of an Asn167 → Ile mutation in a PKU patient (37). Moreover, Asn207 and Asn223 also seem to have a structural role in hPAH. The substitution of Asn207 and Asn223 by Asp residues resulted in a decreased kinetic cooperativity of L-Phe binding and no activation by the pterin cofactor (Table III). Thus, the kinetic properties of the recombinant N167I tetramer do not explain why the mutation is associated with PKU. However, some PKU-related mutant forms are characterized by their reduced cellular stability (20, 26, 40), and degradation of misfolded mutant proteins has been proposed as a general mechanism whereby a missense mutation leads to PKU/hyperphenylalaninemia (20, 26, 40). The N167I mutant gave a low recovery of the tetrameric form on size-exclusion chromatography after cleavage of the fusion protein (data not shown) due to aggregation, and the steady-state kinetics were therefore determined using the tetrameric fusion protein. An isoleucine at this position may destabilize the secondary structure (Fig. 5), and the presence of a hydrophobic residue on the outside of the α-helix, accessible to the solvent, may explain the tendency of the N167I mutant form to aggregate.

The Enzyme Phenotype of the Disease-associated Mutations Asn167 → Ile and Asn207 → Asp—More than 400 mutations associated with PKU/hyperphenylalaninemia have been identified so far in the PAH gene (39) (data.mch.mcgill.ca/pahdb_new), but only three of them have been reported in the codons for Asn residues, i.e., N167I, N207D, and N207S. The N167I mutation has been found in a few PKU patients in Wales, Scotland, and Belgium (37), but so far no information has been reported on their metabolic and enzymatic phenotypes. Compared with wt-hPAH (2-h induction at 28 °C), the N167I mutation revealed an almost 2-fold higher catalytic efficiency and a higher Hill coefficient for the L-Phe binding, although the apparent V_max was reduced by about 50% (Table III). In addition, the K_m value for the pterin cofactor was decreased (Table III). Thus, the kinetic properties of the recombinant N167I tetramer do not explain why the mutation is associated with PKU. However, some PKU-related mutant forms are characterized by their reduced cellular stability (20, 26, 40), and degradation of misfolded mutant proteins has been proposed as a general mechanism whereby a missense mutation leads to PKU/hyperphenylalaninemia (20, 26, 40). The N167I mutant gave a low recovery of the tetrameric form on size-exclusion chromatography after cleavage of the fusion protein (data not shown) due to aggregation, and the steady-state kinetics were therefore determined using the tetrameric fusion protein. An isoleucine at this position may destabilize the secondary structure (Fig. 5), and the presence of a hydrophobic residue on the outside of the α-helix, accessible to the solvent, may explain the tendency of the N167I mutant form to aggregate. Because codon 167 is located at the end of exon 5 in the hPAH gene, one should also consider the possibility that the mutation may affect the splicing of this exon. The N207D mutation has been identified in a Korean PKU patient as a compound heterozygote with the mutant allele Y325X (41), but no information has yet been reported on the metabolic and enzymatic phenotype of this heteroallelic genotype. The recombinant N207D mutant tetramer revealed a decreased V_max value as well as a decreased affinity for L-Phe and an increased affinity for the pterin cofactor relative to wt-hPAH, but it did not show any kinetic cooperativity of L-Phe binding and no activation by L-Phe preincubation. Furthermore, the dramatic effect shown on tryptic proteolysis and temperature stability by the isolated tetrameric mutant enzyme indicates that the mutant protein is in a rather open conformational state and thus more susceptible to intracellular proteolysis than the wild-type hPAH. Thus, the expected enzymatic and metabolic phenotype of the N207D/ Y325X alleles is a classic PKU. The Asn167 is located in the middle of the Co4-helix of hPAH and is a highly conserved
residue in PAH of different species, also suggesting an important structural role. It is stabilized by a hydrogen bond between Asn$^{108}$N$\equiv$O2 and Ala$^{205}$ carbonyl oxygen (3.13 Å) (located in the loop between Co3-helix and Co4-helix) and an electrostatic attraction to Tyr$^{198}$ carbonyl oxygen (3.6 Å) (located in the end of Co3-helix) (Fig. 5). The negative charge on the mutant Asp residue is likely to induce an electrostatic repulsion with both the carbonyl groups of Ala$^{202}$ and Tyr$^{198}$ (Fig. 5) and therefore disrupt the tertiary structure in this region by moving the α-helices away from each other.

Concluding Remarks—Post-translational modifications of proteins are important in the regulation of many cellular processes, providing a way to specifically change the structure and function of target proteins. Although phosphorylation and acetylation have received the most attention, nonenzymatic deamidation of labile Asn residues is also a frequently occurring post-translational modification in proteins (42), as first shown for the human phenylalanine hydroxylase (BH3 domain-only proteins). Equally interesting was the finding that deamidation was observed to be suppressed by the interaction of Bcl-X$\alpha$ with a specific retinoblastoma protein. Thus, specific protein-protein interactions may represent a third structural factor in determining the rate of deamidation of Asn residues in target proteins in vivo.

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