Posttranslational Hydroxylation of Human Phenylalanine Hydroxylase Is a Novel Example of Enzyme Self-Repair within the Second Coordination Sphere of Catalytic Iron

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Phenylalanine hydroxylase, a mononuclear non-heme iron enzyme, catalyzes the oxidation of phenylalanine to tyrosine in the presence of oxygen and reduced pterin cofactor. X-ray structural studies have established the coordination around the iron and point to significant interactions within the second coordination sphere. One such interaction in human phenylalanine hydroxylase (hPAH) is the focus of this report. Residue Tyr325 is part of the hydrogen-bonding network around the binding cleft, the so-called second shell (Figure 1). It is hydrogen-bonded through its hydroxyl to a water molecule that coordinates to both iron and pterin, thereby taking part of a network that appears important for the stabilization of the binding site.[1,2] Despite this apparent role of Tyr325 in positioning the pterin, its role in catalysis had been dismissed as it was shown that the conservative mutation of Tyr325 to Phe325 had no effect on the observed enzymatic activity.[3,4] The interpretation of this result is unlikely considering that the loss of the hydroxyl from the catalytic site could destabilize the coordination around iron.

In this study, both full-length tetramer (1−452) and truncated dimer (117−424) Tyr325Phe hPAH mutant enzymes showed kinetics, thermal stabilities, and oligomerization profiles similar to those of their corresponding wild-type (Wt) enzymes. Meanwhile, the equivalent mutation Tyr179Phe in the bacterial form of this enzyme from *Chromobacterium violaceum*, cPAH, showed 30-fold kinetic reduction in k_{cat} as compared to wild-type cPAH.[5] This intriguing difference in the kinetics between the mutant hPAH and the mutant cPAH indicated to us the importance of Tyr325 and the possible selective hydroxylation of Phe325 occurring in vivo as an adaptive response that restores the damage.[6] Therefore, we immediately examined a possible in vivo posttranslational hydroxylation by mass spectrometry on digested mutant and wild-type hPAH enzymes to provide an indisputable answer.

Wild-type and Tyr325Phe mutant hPAH proteins were both expressed as MBP-hPAH1−452 and MBP-hPAH117−424 maltose-binding fusion proteins.[7] Hydroxylation of hPAH mutant was supported by ESI-MS of the intact protein and MALDI-TOF of the tryptic digest (Figure S1A,B). The Tyr179Phe cPAH mutant that was clearly less active than Wt cPAH displayed a mass of 16 Da less than the wild-type.[5] The kinetics of Wt and mutant hPAH enzymes indicated similar activities (k_{cat}) and affinities (K_m) for phenylalanine and pterin (Table S1). While the k_{cat} values for wild-type 1−452 and Tyr325Phe were identical, k_{cat} for wild-type 117−424 was 14.3 and 5.2 s^{-1} for Tyr325Phe (Table S1). This 3-fold reduction in the k_{cat} for the catalytic domain mutant enzyme reveals the additional importance of the regulatory and tetramerization domains in accomplishing the hydroxylation and recovery of activity. The similarity between wild-type 1−452 and mutant
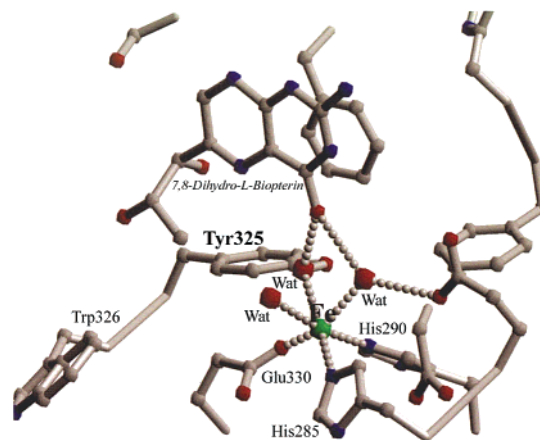

Figure 1. hPAH active site highlighting the hydrogen-bonding network involving Tyr325.

Tyr325Phe was further supported by the oligomerization status of both proteins. The 1−452 Tyr325Phe mutant enzyme revealed a functional tetrameric fraction similar to that of the wild-type as shown in the ratio of aggregate to tetramer (Figure S2). On the other hand, the completely inactive Tyr325Ala mutant failed to express as a tetramer and was completely aggregated, which underscores the importance of Tyr325 in the folding and catalysis of the enzyme. Moreover, the melting temperatures of both Tyr325Phe mutant and Wt enzymes were identical, confirming similar thermal stabilities as shown by DSC (Table S2 and Figure S3).

For the mass spectrometry analysis, tryptic peptides of wild-type and mutant hPAH were generated by in-gel digestion.[8,9] Briefly, the tetrameric form of the enzyme was run on SDS polyacrylamide gel electrophoresis and visualized with Coomassie. Protein bands were excised, destained with a mixture of 50 mM ammonium bicarbonate and acetonitrile (1:1, v:v), and treated with 10 mM DTT. Subsequently, cysteines were derivatized with iodoacetamide, and the gel-entrapped protein was digested with trypsin. Resulting peptides were extracted twice with 60% acetonitrile/1% TFA, and the combined extracts were evaporated to dryness in a vacuum centrifuge. The dried samples were dissolved in 10 μL of 50% acetonitrile, 1% formic acid, and subjected to an Applied Biosystems QStar Pulsar i instrument for ESI-QqTOF-MS and MS/MS analysis of doubly charged molecules of target tryptic peptides. The ESI-MS spectrum of 1−452 Tyr325Phe shows the relative abundance of the doubly charged precursor ions at m/z 924.4 and 916.5 (Figure S4) of the peptides 321−335 (Table S3) with and without hydroxylation, respectively, and their product ion spectra were recorded at collision offset voltages of 40 V. The
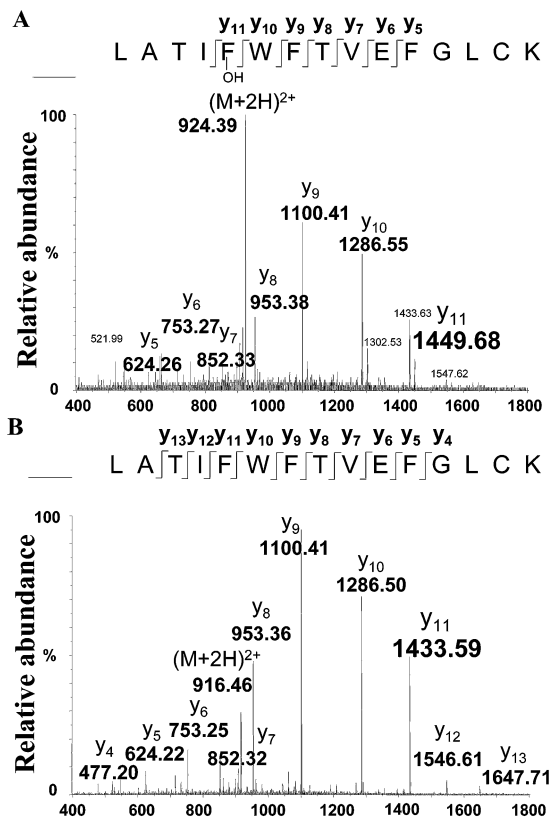
Figure 2. Tandem mass spectrometry of tryptic peptides from mutant Tyr325Phe hPAH (1−452). (A) ESI-MS/MS spectrum of $(M + 2H)^{2+}$ m/z 924.4 derived from HO-Phe325 hydroxylated mutant. (B) ESI-MS/MS spectrum of $(M + 2H)^{2+}$ m/z 916.5 derived from Phe325 mutant.

obtained amino acid tags confirmed the presence of Phe325 in the peptide corresponding to the doubly charged precursor ion at m/z 916.5 (L A T I F W F T V E F G L C K), and of hydroxylated Phe325 in the peptide corresponding to the m/z 924.4 byproduct ion series comprising the fragments y_5-y_{12} (Figure 2). Attention is focused on the product ions y_{10} at m/z 1286.5 and y_{11}, the latter of which is found at m/z 1433.6 in the presence of Phe325 (Figure 2B) but which shifts by 16 Da to m/z 1449.7 due to hydroxylation of Phe325 (Figure 2A), thereby resembling the wild-type enzyme. In addition to Phe325 hydroxylation, indication for Trp326 (Figure 1) hydroxylation is observed with a split of the fragment ion y_{10} occurring with a mass difference of 16 Da at m/z 1286.6 and 1302.5. The hydroxylation in the mutant is not quantitative because both the Tyr325Phe point mutant and the Wt hPAH in our hands display only ∼50% metalation as shown by ICP-AE. Moreover, this observation demonstrates a self-hydroxylation mechanism that is dependent on iron in the active site. The presence of a peak at m/z 1433.6 in Figure 2A does not exclude a possible hydroxylation at any of the residues 321−324. The latter possibility is unlikely due to their distant positioning from the active site and the absence of hydroxylation in these residues in other controls (Wt and Tyr325Ala mutant hPAH). Interestingly, the catalytic domain 117−424 of this mutant enzyme also becomes hydroxylated, attesting to the importance of Tyr325 in stabilizing the active site (Figure S5C,D). Meanwhile, the Tyr325Ala mutant does not feature a phenyl ring and displays y_{11} at m/z 1357.7, confirming the presence of Ala325 (Figure S5A).

The hydroxylation as confirmed by MS/MS data occurred in vivo as a posttranslational modification of Phe325 without requiring oxidizing conditions. This is in contrast to the in vitro hydroxylation of Phe300 in wild-type tyrosine hydroxylase that takes place only in the presence of excess pterin, iron, and DTT.[10,11] Other accounts of hydroxylation include Trp112 in α-KG-Fe(II)TfdA, causing subsequent inactivation of TfdA, due to HO-Trp coordination to iron, and TauD modification.[12] Self-hydroxylation of a mutant ribonucleotide reductase ApoR2 F208Y is caused by in vitro addition of ferrous iron to form DOPA-208.[13] The self-hydroxylation of Phe325 herein is a novel example of posttranslational modification that takes place in vivo and results in enzyme repair rather than damage. In support of the biological significance of Phe325 hydroxylation, aspartyl β-hydroxylation of EGF domains of Notch receptor proteins, asparaginyl hydroxylation of HIF transactivation domain in normoxic cells, and prolyl hydroxylation of HIF are all events that occur as posttranslational modifications.[14] The reported "uncoupled" self-hydroxylation of hPAH uncovers a biochemical significance that may serve to protect the enzyme from a nonselective and irreversible oxidation that is more damaging. We have shown that Tyr325 plays an important role structurally and in catalysis, thereby allowing Phe325 hydroxylation in a repair mechanism to retain wild-type function.

Supporting Information Available: Protein expression, ESI-MS on hPAH and digests, MALDI-TOF on Y325F tryptic digest, HPLC and DSC profiles, melting temperatures, and ESI-MS and MS/MS spectra for Y325A, Wt (117−424), Y325F, and tryptic map (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Erlandsen, H.; Fusetti, F.; Martinez, A.; Hough, E.; Flatmark, T.; Stevens, R. C. *Nat. Struct. Biol.* **1997**, *4*, 995−1000.
(2) Erlandsen, H.; Kim, J. Y.; Patch, M. G.; Han, A.; Volner, A.; Abu-Omar, M. M.; Stevens, R. C. *J. Mol. Biol.* **2002**, *320*, 645−661.
(3) Erlandsen, H.; Bjorgo, E.; Flatmark, T.; Stevens, R. C. *Biochemistry* **2000**, *39*, 2208−2217.
(4) The equivalent mutation of Tyr371 to Phe371 in the structurally analogous rat tyrosine hydroxylase had no effect on the catalytic activity. Daubner, S. C.; Fitzpatrick, P. F. *Biochemistry* **1998**, *37*, 16440−16444.
(5) Zoidakis, J.; Abu-Omar, M. M., personal communication.
(6) Squier, T. C. *Exp. Gerontol.* **2001**, *36*, 1539−1550.
(7) Martinez, A.; Knappskog, P. M.; Olafsdottir, S.; Doskeland, A. P.; Eiken, H. G.; Svebak, R. M.; Bozzini, M.; Apold, J.; Flatmark, T. *Biochem. J.* **1995**, *306*, 589−597.
(8) Shevchenko, A.; Wilm, M.; Vorm, O.; Mann, M. *Anal. Chem.* **1996**, *68*, 850−858.
(9) Van Montfort, B. A.; Canas, B.; Duurkens, R.; Godovac-Zimmermann, J.; Robillard, G. T. *J. Mass Spectrom.* **2002**, *37*, 322−330.
(10) Ellis, H. R.; Daubner, S. C.; McCulloch, R. I.; Fitzpatrick, P. F. *Biochemistry* **1999**, *38*, 10909−10914.
(11) Goodwill, K. E.; Sabatier, C.; Stevens, R. C. *Biochemistry* **1998**, *37*, 13437−13445.
(12) (a) Liu, A.; Ho, R. Y. N.; Que, L., Jr.; Ryle, M. J.; Phinney, B. S.; Hausinger, R. P. *J. Am. Chem. Soc.* **2001**, *123*, 5126−5127. (b) Ryle, M. J.; Liu, A.; Muthukumaran, R. B.; Ho, R. Y.; Koehntop, K. D.; McCracken, J.; Que, L., Jr.; Hausinger, R. P. *Biochemistry* **2003**, *42*, 1854−1862.
(13) (a) Ormo, M.; deMare, F.; Regnstrom, K.; Aberg, A.; Sahlin, M.; Ling, J.; Loehr, T. M.; Sanders-Loehr, J.; Sjoberg, B.-M. *J. Biol. Chem.* **1992**, *267*, 8711−8714. (b) Logan, D. T.; deMare, F.; Persson, B. O.; Slaby, A.; Sjoberg, B.-M.; Nordlund, P. *Biochemistry* **1998**, *37*, 10798−10807.
(14) (a) Dinchuk, J. E.; Focht, R. J.; Kelley, J. A.; Henderson, N. L.; Zolotarjova, N. I.; Wynn, R.; Neff, N. T.; Link, J.; Huber, R. M.; Burn, T. C.; Rupar, M. J.; Cunningham, M. R.; Selling, B. H.; Ma, J.; Stern, A. A.; Hollis, G. F.; Stein, R. B.; Friedman, P. A. *J. Biol. Chem.* **2002**, *277*, 12970−12977. (b) Lando, D.; Peet, D. J.; Whelan, D. A.; Gorman, J. J.; Whitelaw, M. L. *Science* **2002**, *295*, 858−861. (c) Bruick, R. K.; McKnight, S. L. *Science* **2001**, *294*, 1337−1340.

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