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Demonstration of a Peroxide Shunt in the Tetrahydropterin-Dependent Aromatic Amino Acid Monooxygenases

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Abstract

The non-heme iron enzyme phenylalanine hydroxylase, tyrosine hydroxylase, and tryptophan hydroxylase catalyze the hydroxylation of their aromatic amino acid substrates using a tetrahydropterin as the source of electrons. The hydroxylating intermediate is proposed to be an Fe(IV)O species. We report here that all three enzymes will catalyze hydroxylation reactions using H_2O_2 in place of tetrahydropterin and oxygen, forming tyrosine and 3-hydroxyphenylalanine from phenylalanine, 4-$HOCH_2$-phenylalanine from 4-CH_3-phenylalanine, and hydroxycyclohexylalanine from 3-cyclohexylalanine. No peroxide-dependent reaction is seen with active site mutants of TyrH and PheH in which the stability or reactivity of the iron center is compromised. These results provide further support for an Fe(IV)O hydroxylating intermediate.

Phenylalanine hydroxylase (PheH), tyrosine hydroxylase (TyrH) and tryptophan hydroxylase (TrpH) are non-heme iron monooxygenases that catalyze the insertion of an oxygen atom from O_2 into the aromatic side chain of their corresponding substrates using a tetrahydropterin (PH_4) substrate as the reductant.[1–3] Their active sites each contain a mononuclear iron coordinated by two histidines and a glutamate,[4–6] an arrangement that has been termed a 2-his-1-carboxylate facial triad.[7,8] Scheme 1 shows the chemical mechanism proposed for PheH, TyrH, and TrpH.[3] The hydroxylating intermediate is an Fe(IV)O capable of aromatic, benzylic, and aliphatic hydroxylation.[9,10] This species has recently been detected in TyrH by freeze-quench Mössbauer spectroscopy.[11] The spectra and reactivity of the Fe(IV)O intermediate resemble those in members of the α-ketoglutarate-dependent hydroxylase family, which also contain a mononuclear iron coordinated by a 2-his-1-carboxylate facial triad.[12,13]

In Scheme 1 the PH_4 supplies two electrons to reduce one atom of O_2 to the level of water, but it plays no role in the actual oxygen transfer to the amino acid substrate. This suggests that it could be possible to bypass the PH_4 and generate the Fe(IV)O intermediate directly with an alternative oxygen donor. Such a shunt has been possible in the cases of the heme-based cytochrome P450,[14] the binuclear non-heme methane monooxygenase,[15] and mononuclear non-heme dioxygenases,[16,17] but not with a mononuclear non-heme monooxygenase.

We now report that H_2O_2 can replace PH_4 and O_2 to support amino acid hydroxylation by the aromatic amino acid hydroxylases. Incubation of PheH,[18,19] TyrH,[20] or TrpH[19,21] with phenylalanine and H_2O_2 results in the formation of tyrosine and 3-HO-phenylalanine (Figure 1). No hydroxylated amino acids are detectable if apoenzyme is used. The rate of hydroxylation is unchanged when the reaction is carried out in the absence of O_2. The ratio of tyrosine to 3-HO-phenylalanine produced is different for the three enzymes, with ratios of 1.5, 1.2, and 1.6 for PheH, TyrH and TrpH, respectively. Controls showed that the PheH does not lose activity

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under these conditions. No amino acid products could be detected with sodium periodate, cumene hydroperoxide, peracetic acid, or tbutyl hydroperoxide instead of H_2O_2. The yield of hydroxylated amino acids was not affected by the radical quenchers mannitol or benzoate (10 mM) or by 2 mM 5-deaza-6-methyltetrahydropterin.

TyrH has previously been shown to produce tyrosine and 3-HO-phenylalanine from phenylalanine in PH_4-dependent turnover,[22] but PheH and TrpH only produce tyrosine.[18, 21] Thus, the lack of the PH_4 has an effect on the substrate specificity of these enzymes. Tyrosine and tryptophan were also examined as substrates in the peroxide-dependent reactions for TyrH and TrpH, but the expected products 3,4-dihydroxyphenylalanine and 5-HO-tryptophan were not detected. Control reactions showed that these two compounds are not stable to the reaction conditions.

The kinetics of the H_2O_2-dependent reactions were examined for comparison with PH_4-dependent turnover. The initial rate of phenylalanine hydroxylation was directly dependent on the concentration of enzyme (Figure 2). As also shown in Figure 2, the reaction continued for multiple turnovers. When the concentration of H_2O_2 was varied, the initial rate of the reaction for all three enzymes showed saturation kinetics, with K_m values of ~20 mM for each enzyme. In contrast, the initial rate of the reaction did not show evidence for saturation with phenylalanine at concentrations as high as 50 mM for any of the enzymes. The linear dependence of the rate on the concentration of phenylalanine yields k_{cat}/K_{phe} values of 6.4, 5.3, and 4.3 M^{-1} min^{-1} for the H_2O_2-dependent reaction for PheH, TyrH and TrpH, respectively. These values are 5–6 orders of magnitude smaller than the corresponding values for PH_4-dependent turnover.[18,21] As shown in Scheme 1, formation of the Fe(IV)O species is proposed to require heterolytic cleavage of the O-O bond in an iron-peroxo-pterin intermediate, with a HO-pterin as the leaving group. Formation of the Fe(IV)O in the H_2O_2-dependent reaction would be expected to result from loss of water from an iron-peroxide intermediate. The much slower reaction with H_2O_2 is consistent with the different pK_a values of the leaving groups in the H_2O_2 and PH_4-dependent reactions. In addition, the similar kinetic parameters for all three enzymes in the H_2O_2-dependent reactions are consistent with the hydroxylating intermediates having similar reactivity for all three enzymes. The reactivities of the Fe(IV)O intermediates in PH_4-dependent turnover have previously been shown to be similar.[23]

Active site mutants of TyrH and PheH that affect PH_4-dependent turnover were examined in the H_2O_2-dependent reaction. With E332A TyrH, only 2.5% of the reducing equivalents from $6MePH_4$ are used for productive turnover.[20] S395A TyrH forms the 4a-HO-pterin at a normal rate, but the hydroxylating intermediate breaks down unproductively so that only 1% is used to hydroxylate tyrosine.[24] V379D and F263A PheH have low turnover due to uncoupling of PH_4 oxidation and amino acid hydroxylation.[19,25] With all four mutant enzymes no tyrosine or 3-HO-phenylalanine could be detected in the H_2O_2-dependent reactions. Thus, amino acid residues required for proper reactivity of the Fe(IV)O intermediate in PH_4-dependent turnover are also required for H_2O_2-dependent turnover.

The aromatic amino acid hydroxylases have previously been shown to catalyze benzylic[9,23] and aliphatic hydroxylation.[26] To determine if the H_2O_2-dependent reaction is also capable of supporting these nonphysiological reactions, 4-CH_3-phenylalanine and cyclohexylalanine were examined as substrates. In our hands TyrH and PheH catalyze the PH_4-dependent hydroxylation of cyclohexylalanine to form 4-HO-cyclohexylalanine with k_{cat} values of 10 and 5 min^{-1}, respectively, at 30 °C. Both enzymes also catalyze the same reaction using H_2O_2, with second order rate constants of 0.17 M^{-1} min^{-1} and 0.28 M^{-1} min^{-1}. With 4-CH_3-phenylalanine as substrate for PH_4-dependent turnover, all three enzymes produce a combination of 4-CH_2OH-, 3-HO,4-CH_3-, and 4-HO,3-CH_3-phenylalanine.[23] In the H_2O_2-

dependent reactions, 4-CH_2OH-phenylalanine is produced but the other two products could not be detected.

The present results establish that H_2O_2 can replace PH_4 and O_2 to form the hydroxylating intermediate in the aromatic amino acid hydroxylases. The similar kinetic parameters for all three enzymes in the H_2O_2-dependent reactions are consistent with the hydroxylating intermediates having similar reactivity for all three enzymes. These results provide support for a hydroxylating intermediate such as Fe(IV)O that does not involve the pterin.

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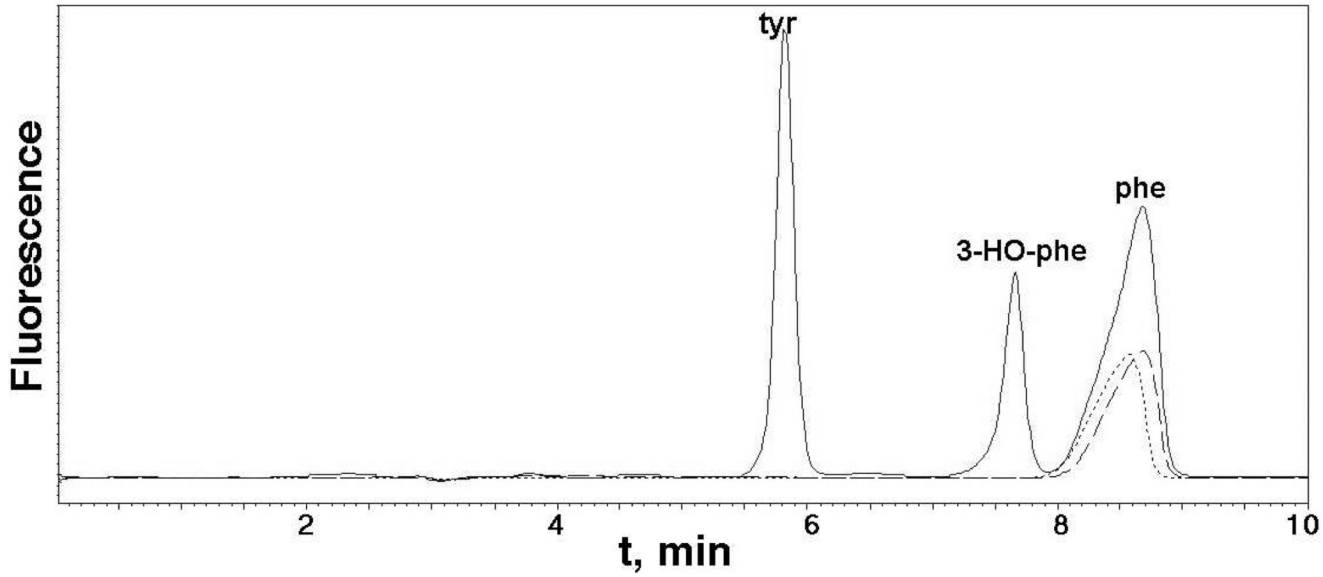


Figure 1.

Peroxide-dependent hydroxylation of phenylalanine by tyrosine hydroxylase. Solid line: TyrH (25 μM) was incubated for 15 min at 30 °C with 10 mM H_2O_2, 400 μM ferrous ammonium sulfate, 20 min phenylalanine, and 100 mM NaCl in 150 mM Hepes buffer, pH 7.0. Dashed line: reaction with 50 μM apo-TyrH. Dotted line: Reaction with 400 μM ferrous ammonium sulfate but no enzyme. The products of the reaction were analyzed by reverse-phase HPLC, using a mobile phase of 15 mM sodium phosphate, pH 7.0, 1% tetrahydrofuran, with excitation at 270 nm and emission at 310 nm.

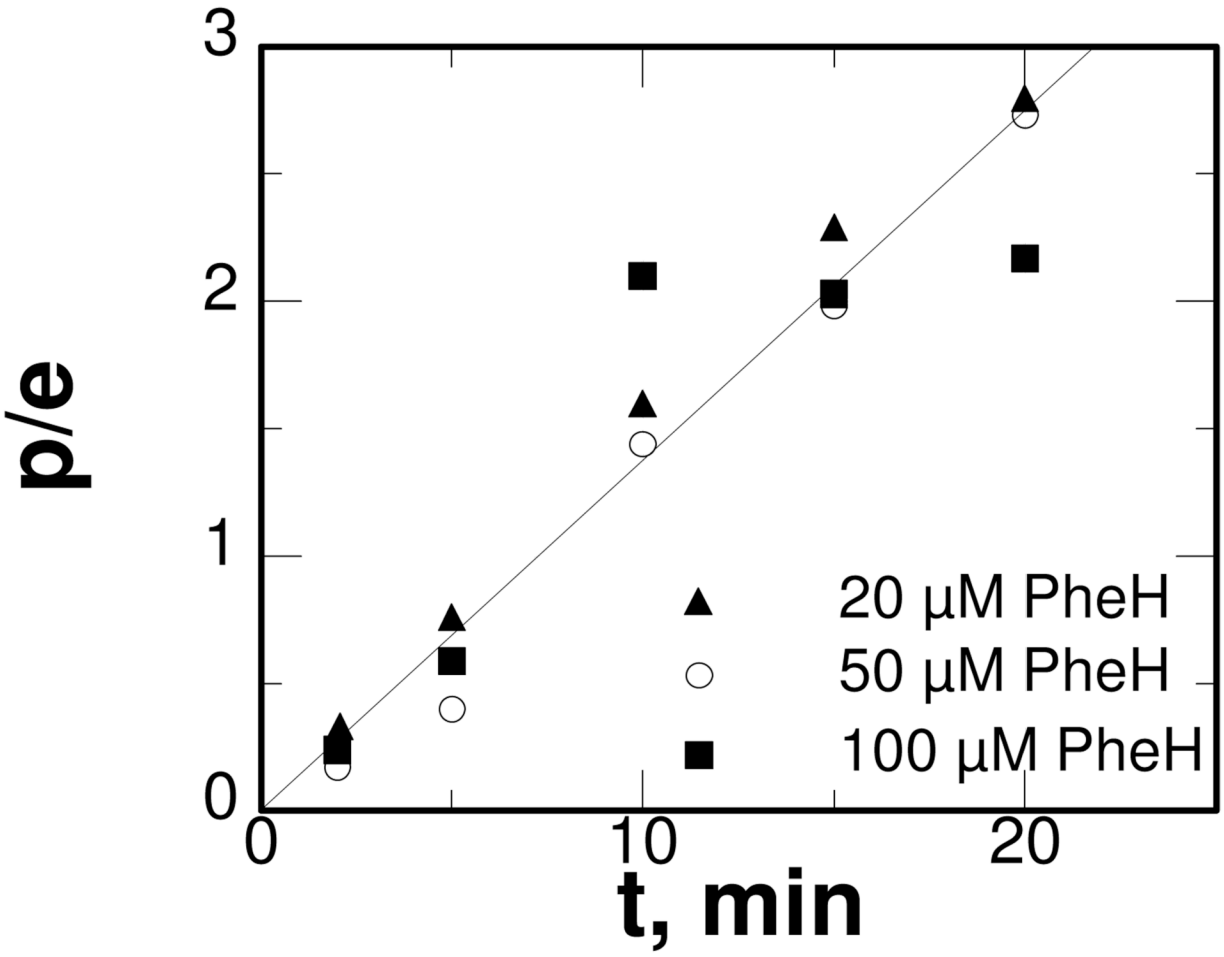
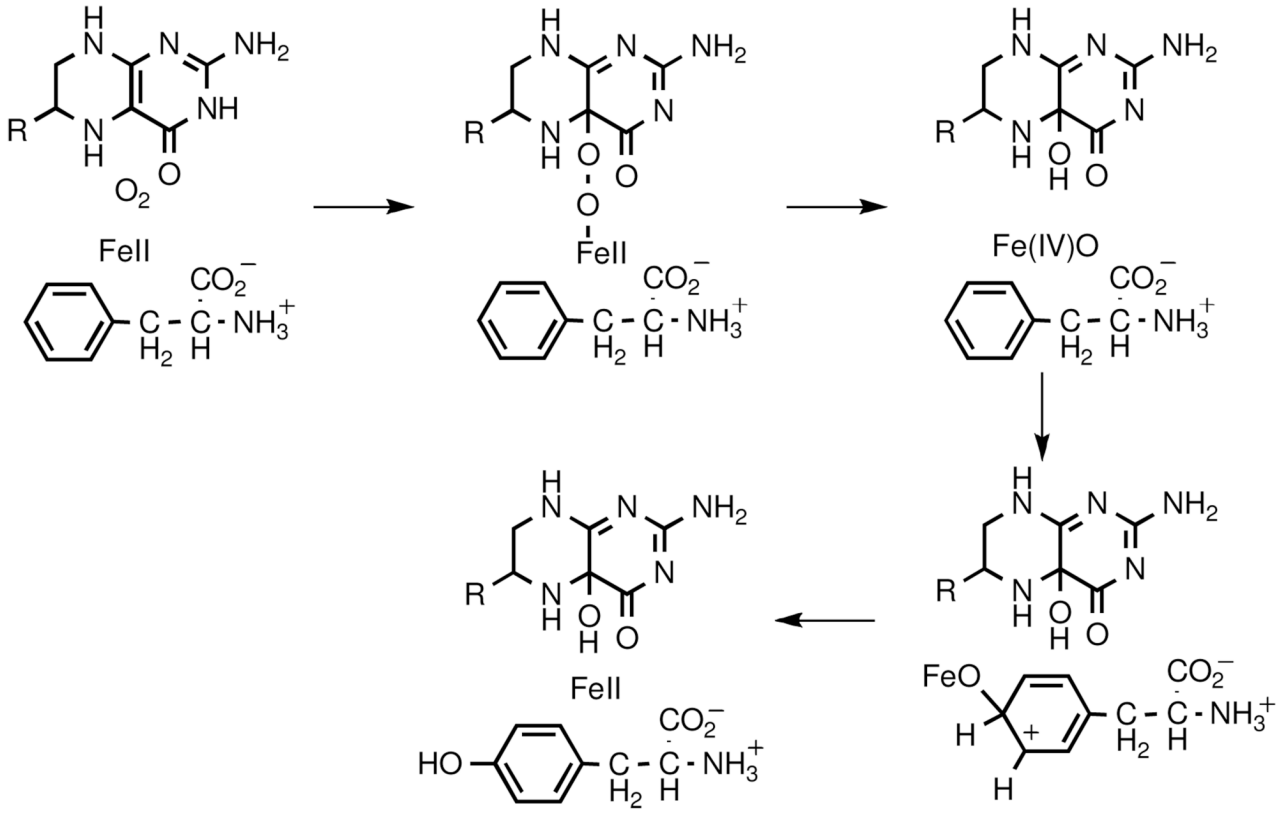


Figure 2.
Dependence of the amount of hydroxylated phenylalanine on the concentration of PheH for the peroxide-dependent reaction; p/e, moles of hydroxylated amino acid per mole of enzyme. Conditions as for Figure 1.



Scheme 1.