The evolutionary constraints imposed on tyrosine hydroxylation by its beginnings as a phenylalanine hydroxylase; studies of the polypeptide loop determining the substrate specificity.

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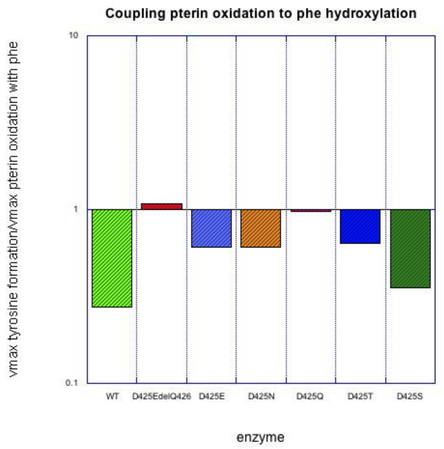
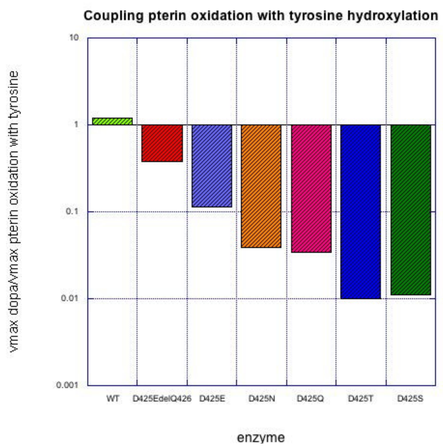
ABSTRACT

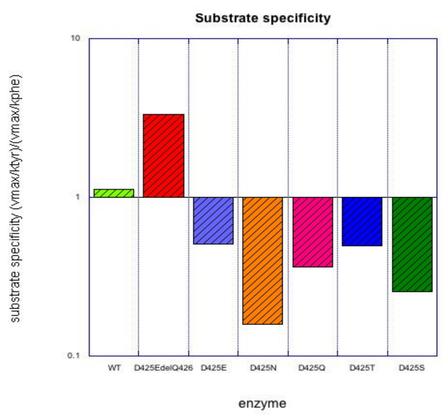
Tyrosine hydroxylase (TyrH), phenylalanine hydroxylase (PheH) and tryptophan hydroxylase (TrpH) belong to the family of aromatic amino acid hydroxylases (AAAH) and are genetically related. An ancient PheH probably was the ancestor of the modern enzymes. Their active sites are very similar, however their substrate specificities vary. They all hydroxylate phenylalanine; TyrH is the only one that can make DOPA from tyrosine. Previous work identified the aspartate at position 425 in TyrH, which lies in a very flexible and dynamic polypeptide loop, to be critical for the enzyme's achievement of this new activity. We started this project to examine whether alterations in the shape of this polypeptide loop could make a stronger TyrH. The crystal structure of TyrH shows D425 is at the end of this loop. We also knew that substituting it with a glutamate (D425E) decreased its DOPA forming ability 10-fold. We sought to find out if making the loop shorter but keeping the glu would make a strong TyrH. Therefore, we deleted separately and together the glutamines (Q424 and Q426) that are adjacent to D425. Of these three mutants, only D425EΔQ426 has comparable activity to WT. Previously reported V_{max} values are: WT, 150; D425E TyrH, 13.4. The value for D425EΔQ426 is 118. We determined the specific activity for the enzyme by finding the ratio of V_{max}/K_M for tyrosine to V_{max}/K_M for phenylalanine and comparing this to WT's and previous mutants' values. The value for D425EΔQ426 was 3.3, and for WT, 3.75, and for D425E TyrH, 0.34.





COUPLING

Coupling refers to the simultaneous oxidation of one 6-MeBH$_4$ molecule for each molecule of substrate that is hydroxylated. The oxidation rates were determined using a coupled assay with DHPR. By monitoring how much NADH is oxidized we could measure how much pterin is oxidized and how much substrate is hydroxylated.



Tables
1- Steady-State Kinetics Parameters for tyr, phe hydroxylation, pterin oxidation,

Enzyme	K_{tyr} from DOPA	V_{max}DOPA	K_{phe} from Tyr formation	V_{max} Tyr form
wild-type	40 +/- 4	200 +/- 13	100+/- 15	96+/- 12
D425EΔQ426	9.6+/- 6.9	38.3+/- 8.1	1.7+/-1	119.2+/- 13
D425E	97 +/-10	13.4 +/- 0.4	45+/- 11	76+/- 13
D425N	172+/-27	4.6 +/- 0.2	62+/- 14	162+/-5
D425Q	162+/- 32	7.3 +/- 0.4	56.5+/- 9.4	349+/- 16
D425T	-	0.44 +/- 0.023	16+/- 5	141+/- 2
D425S	-	1.01 +/- 0.007	9.2+/- 4.6	31.6+/- 4.5

Enzyme	K_{tyr}ptetinoxidation with tyr	V_{Max}pterinoxidation withtyr	V_{max}/ K_{tyr}pterinoxidation with tyr
wild-type	42 +/- 9	165 +/- 11	3.929
D425EΔQ426	5.6+/- 1.5	100 +/- 6.8	17.86
D425E	83.3 +/- 33	119 +/- 12.2	1.429
D425N	172 +/- 27	117+/- 5.8	0.6802
D425Q	137 +/- 26	214 +/- 12.6	1.562
D425T	1.05 +/- 0.22	44.2 +/- 1	42.1
D425S	8.9 +/- 1.6	91.7 +/- 2.5	10.3

Enzyme	K_{phe}pterinoxidation with phe	V_{Max}pterinoxidation withphe	V_{max}/ K_{phe}pterinoxidation with phe
wild-type	100 +/- 22	349 +/- 22	3.49
D425EΔQ426	20.5+/- 7	110.2+/- 11.4	5.3756
D425E	44.6 +/- 11	125 +/- 5	2.8027
D425N	62 +/- 13.6	266 +/- 10	4.2903
D425Q	84+/- 13	358 +/- 17	4.2619
D425T	2.6 +/- 0.9	221 +/- 12	85
D425S	2.2 +/- 0.47	89.4 +/- 3.5	40.636

Enzyme	Substratespecificity (v_{max}/K_{tyr})/ (v_{max}/K_{phe})	V_{max}DOPA/v_{max} pt ox with tyr	V_{max}Tyr form/ V_{max} pt ox with phe
wild-type	1.1257	1.2121	0.27507
D425EΔQ426	3.3219	0.383	1.0817
D425E	0.50971	0.11261	0.608
D425N	0.15855	0.039316	0.60902
D425Q	0.36651	0.034112	0.97486
D425T	0.49524	0.0099548	0.63801
D425S	0.25355	0.011014	0.35347

BACKGROUND INFORMATION

Tyrosine hydroxylase (TyrH), phenylalanine hydroxylase (PheH) and tryptophan hydroxylase (TrpH) belong to the family of aromatic amino acid hydroxylases (AAAH) and are genetically related. Their active sites are very similar, however their substrate specificities vary. TyrH is able to hydroxylate all three amino acids phe, tyr and trp. However, PheH and TrpH can only hydroxylate phe and trp (Fig. 1). They all catalyze the rate limiting steps for different pathways. TyrH is involved in the catecholamine synthesis (Fig. 2), PheH in phenylalanine catabolism and TrpH in serotonin synthesis[1].TyrH plays an important role in the nervous system and its deficiency can lead to a number of neurological disorders such as Parkinson's disease and addictive behavior. It catalyses the hydroxylation of tyrosine to DOPA, which is then converted to dopamine and other catecholamines. Previous work identified the aspartate at position 425 in TyrH, which lies on a very flexible and dynamic polypeptide loop (Fig. 3), to be critical for the enzyme's achievement of the evolutionarily new ability to synthesize DOPA from tyrosine[2]. This amino acid residue lies over the opening of the active site in that flexible loop region. It is believed that its role is to orient the substrate in space in such a way to allow for the electrophilic substitution at the aromatic ring. The D425 was previously determined to be critical for the enzyme's substrate specificity and mutagenesis of that residue severely alters the activity of the enzyme. Previous work showed that D245E mutant was one of the few that were able to synthesize DOPA but only 10% as well as wild-type.

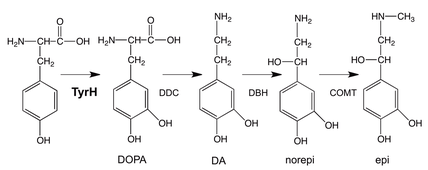


Figure 2. Catecholamine synthesis pathway.

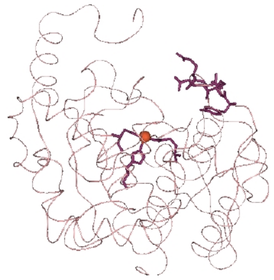


Figure 3. circle fe held down by 3 aa. D425 region in TyrH. Spec loop in upper right, 5 aa tyr, glu, asp, glu, threo.

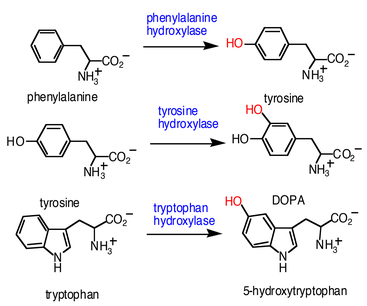


Figure 1.

RESULTS

Using the V_{max}, K_{tyr} and K_{phe} values we calculated the ratio of V_{max}/K_M values which gave us the substrate specificity for the enzyme. We compared the data obtained for D425EΔQ426 to the previously reported values for WT and D425E. V_{max} values were WT, 150; D425ETyrH, 13.4; and D425EDQ426, 118, while the specific activity value for D425EΔQ426 was 3.3; WT, 3.75; and D425E TyrH, 0.34. D425EΔQ426 isn't as efficient at coupling as WT is but it still has stronger specificity for tyr than even WT does.

Previous work showed that TyrHD425N and TyrHD425Q had lower activity of DOPA formation compared to WT TyrH but it was high enough to be detected using the standard colorimetric assay. Also, TyrHD245S and TyrHD425T were only able to synthesize DOPA to a small extent, however they could not readily hydroxylate phenylalanine. We believe this is because the hydroxyl groups in these residues can form hydrogen bonds with a water molecule necessary for binding but detrimental to coupling.. These bonds can orient the water in a way that is right for tyrosine but not for phenylalanine hydroxylation

In the future we plan to continue this project with TyrHD425NΔQ426, TyrHD425QΔQ426, TyrHD425SΔQ426 and TyrHD425TΔQ426 and to compare the results to our values from experiments with TyrHD425EΔQ426.

MATERIALS AND METHODS

We mutated, grew and purified the D425EΔQ426, D425EΔQ424 with Q426 and Q424 removed, respectively and a double deletion mutant D425EΔQ424ΔQ426. We used Escherichia coli strain BL21DE3 pLysS for expression of TyrH and pETYH8 plasmid for expression of rat WT TyrH. We first grew all the bacteria similarly for protein expression, that is, at a temperature of 37 ºC prior to induction, followed by a further 3 hours of incubation. This protocol was successful for wild-type TyrH and for D425EΔQ426. Lower but measurable levels of protein were obtained for D425EΔQ424, but we did not obtain large quantities of protein after purification. Practical levels of D425EΔQ424 ΔQ426 in soluble form were not obtainedeither by growth at 37 C or by post-induction growth overnight at 18 ºC. The protein purification was performed on a GE Healthcare AKTA FPLC system using HiTrap heparin. While we were able to express them all, a measured by enzyme activity in clarified lysates, however only D425EΔQ426 had comparable activity to WT and was expressed in large quantities.

We determined the V_{max} and K_M values using four enzyme assays. Formation of DOPA from tyrosine and K_{tyr} value was measured using the standard colorimetric assay and monitoring an increase in absorbance at 490 nm indicating DOPA formation. The amount of DOPA synthesized by the enzyme was determined by comparison to the standard DOPA curve in the assay.

Determination of 6-methyltetrahydropterin (6-MeBH$_4$) oxidation rate was performed using a coupled assay with dihydropteridine reductase (DHPR) which served as a tool allowing for observing the decrease in absorbance at 340 nm due to NADH oxidation. This allowed us to obtain the K_{tyr} and K_{phe} values. The assays were repeated with phenylalanine and tyrosine as a substrate and the hydroxylation of the amino acids was coupled with pterin oxidation.

Formation of tyrosine from phenylalanine was measured using a continuous assay and observing an increase in absorbance at 280 nm due to tyrosine formation. We used Kaleida Graph Synergy software for our calculations.

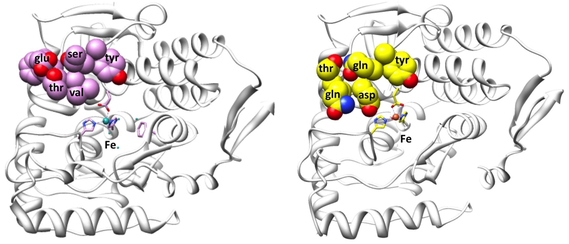


Figure 4. Flexible loop region, PheH shown on the left, TyrH on the right.[3]

REFERENCES

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