THE EFFECT OF VARIATION OF COFACTOR AND SUBSTRATE STRUCTURE ON THE ACTION OF PHENYLALANINE HYDROXYLASE

C.B. Storm and S. Kaufman

Laboratory of Neurochemistry
National Institute of Mental Health
Bethesda, Maryland 20014

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The enzymatic conversion of phenylalanine to tyrosine has been formulated as shown in equations 1 and 2, where XH_4 stands for tetrahydropteridine and XH_2 for quinonoid dihydropteridine (Kaufman, 1964).

$$XH_4 + O_2 + \text{phenylalanine} \rightarrow XH_2 + H_2O + \text{tyrosine} \quad (1)$$

$$XH_2 + TPNH + H^+ \rightarrow XH_4 + TPN^+ \quad (2)$$

Reaction 1 is catalyzed by phenylalanine hydroxylase ("rat liver enzyme") and reaction 2 by dihydropteridine reductase ("sheep liver enzyme"). Consistent with this formulation, it has been found that one mole of XH_4 (or TPNH) is oxidized for each mole of tyrosine formed (Kaufman, 1959).

In 1961, it was reported that phenylalanine hydroxylase could catalyze the hydroxylation of 4-fluorophenylalanine to tyrosine and F^-. Instead of the expected TPNH/tyrosine ratio of two (one extra reducing equivalent is required for the reductive elimination of the fluoride), however, a ratio of about three was observed (Kaufman, 1961).

We would like to report here that the non-stoichiometric use of XH_4 (or TPNH) may be induced by changes in the structure of either the substrate or the pteridine cofactor. Furthermore, it has been shown that with these altered sub-

strates and cofactors, the extra consumption of electrons is due to the hydroxylase catalyzed reduction of oxygen to peroxide.

Materials and Methods

L-phenylalanine and L-tryptophan were obtained from Nutritional Biochemical Company. Biopterin, 6-methylpterin, and 7-methylpterin were prepared by modification of standard methods.* The compounds were characterized by U.V., N.M.R., and mass spectroscopy, as well as by comparison to authentic samples provided by Smith Kline and French and Lederle Laboratories. The pterin was purchased from Calbiochem. Catalase and horse radish peroxidase were purchased from Worthington Biochemical. The pterins were reduced in 0.1 N HCl in a hydrogen atmosphere over PtO catalyst. They were lyophilized and stored as their hydrochlorides at -20°C. Shortly before use, a portion of the tetrahydropterin was dissolved in .005 M HCl and its concentration was determined spectrophotometrically. The rat liver phenylalanine hydroxylase and sheep liver dihydropterin reductase were purified according to the method of Kaufman (1962). The TPNH consumption was assayed spectrophotometrically. Tyrosine and 5-hydroxytryptophan formation was measured by the nitrosonapthol method (Udenfriend et al., 1955, 1952). Oxidation of the tetrahydropterins was measured by titration with 2,6-dichlorophenolindophenol (Kaufman, 1959).

Results and Discussion

The effects of variation of cofactor and substrate

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structure on the action of phenylalanine hydroxylase are given in Table I.

Table I.

Tetrahydropterin	Substrate	μmoles TPNH oxidized / μmoles tyrosine formed	
		Theory	Found
6 methyl	phenylalanine	1.0	.99(.01)[a]
6 methyl	4-F-phenylalanine	2.0	3.44(.23)[b]
7 methyl	phenylalanine	1.0	2.96(.60)
7 methyl	4-F-phenylalanine	2.0	2.91(.66)
pterin	phenylalanine	1.0	2.97(.17)
biopterin	phenylalanine	1.0	.97(.04)
biopterin	4-F-phenylalanine	2.0	2.2
6 methyl	tryptophan	1.0	3.2

a. Standard deviation given in brackets where warranted.

b. Phosphate buffer used instead of tris in this case.

The complete system contained in a final volume of 1 ml the following components, in μmoles: tris - HCl buffer pH 6.8, 100; amino acid, 1-10; TPNH, 0.15; tetrahydropterin, 0.03-0.15; rat liver enzyme 0.06-0.4 mg protein; sheep liver enzyme, 0.5 mg protein; catalase, 0.04 mg protein. The values for TPNH oxidized have been corrected for any oxidation that occurred in the absence of amino acid or hydroxylase.

These results clearly indicate that the efficiency with which the phenylalanine hydroxylase system fuctions is dependent on the structure of both the cofactor and amino acid participating in the reaction.

Molecular oxygen is the most likely acceptor for the extra electrons being consumed under conditions where the altered stoichiometry has been observed. The oxygen could be reduced either to H_2O or to H_2O_2. The following results indicate that H_2O_2 is the product of oxygen reduction under these conditions.

To detect H_2O_2 production, the hydroxylase system was coupled to peroxidase. Preliminary experiments showed that peroxidase catalyzes the H_2O_2 mediated oxidation of XH_4 to quinonoid XH_2. Akazawa (1958) has shown that peroxidase can catalyze the oxidation of reduced pyridine nucleotides by H_2O_2.

As shown in Table I, with phenylalanine and 7-methyltetra-

hydropterin, the ratio of TPNH oxidized to tyrosine formed is close to three. If under these conditions, all of the extra electrons consumed were used to reduce oxygen to H_2O_2, each three moles of XH_4 oxidized would lead to the formation of one mole of tyrosine and two moles of H_2O_2. In the presence of peroxidase, therefore, the expected ratio would be about five. Similar reasoning may be applied in the 4-F-phenylalanine case. As can be seen in Table II the observed ratios in the presence of peroxidase are close to the expected values. The difference may be due to error in determining the small amount of tyrosine formed or may indicate that secondary reactions are taking place.

Table II

Tetrahydropterin	Substrate	μmoles TPNH oxidized / μmoles tyrosine formed	
		Theory	Found
6 methyl	phenylalanine	1.0	1.11(.08)[a]
7 methyl	phenylalanine	4.92	5.31(.60)
6 methyl	4-F-phenylalanine	4.88	5.11(.51)[b]

a. Standard deviation given in brackets.

b. Phosphate used instead of tris in this case.

The complete system is the same as given in Table I except 0.05 mg of horseradish peroxidase substituted for catalase.

These results prove that phenylalanine hydroxylase can function as a classical electron transfer oxidase, in addition to its functioning as a mixed fuction oxidase. They also indicate that the reduction of oxygen to the level of peroxide is a step in the enzyme catalyzed hydroxylation reaction.

Evidence in support of the idea that oxygen at the reduction level of peroxide is a normal intermediate in the hydroxylation reaction was obtained from a study of the rate of peroxide formation as a function of amino acid and tetrahydropterin concentration. As has been discussed before, peroxide formation is due to the non-stoichiometric oxidation of XH_4. Under these

conditions, therefore, the rate of extra TPNH oxidation is a measure of the rate of peroxide formation.

As can be seen from Table III, the K_m values for amino acid substrate and for the tetrahydropterins are the same when the rates of either tyrosine formation or TPNH oxidation are measured, i.e., the rate of hydroxylase-catalyzed formation of peroxide responds to changes in phenylalanine and tetrahydropterin concentration in exactly the same way as does the rate of tyrosine formation.

Table III

Tetrahydropterin	K_m μmole/ml Tyrosine formed	TPNH used	Substrate	K_m μmole/ml Tyrosine formed	TPNH used
7 methyl[a]	.059	.059[c]	phenylalanine	.61	.61
7 methyl[b]	.062	——[c]	phenylalanine	.64	.64
6 methyl[a]	.042	.042[c]	4-F-phenylalanine		
6 methyl[b]	.046	——[c]	4-F-phenylalanine		

a. Catalase: Complete system as given in Table I.

b. Peroxidase: Complete system as given in Table II.

c. Because of competition of the tetrahydropterin at high concentration for the H_2O_2 in a way that was not coupled to TPNH oxidation these two K_m's could only be determined by the rate of tyrosine formation.

Based on the present findings the mechanism of the enzyme-catalyzed hydroxylation reaction can be formulated as shown in equations 3, 4, and 5;

$$E + XH_4 + RH \rightarrow E(XH_4, O_2, RH) \quad (3)$$

$$E(XH_4, O_2, RH) \rightarrow E(XH_2, O_2^{\cdot-}, 2H^+, RH) \quad (4)$$

$$E(XH_2, O_2^{\cdot-}, 2H^+, RH) \begin{cases} \xrightarrow{a} E + XH_2 + RH + H_2O_2 \\ \xrightarrow{b} E + XH_2 + ROH + H_2O \end{cases} \quad (5)$$

where RH = amino acid substrate.

Our results require all three substrates to be present on the enzyme at the time of hydroxylation and oxygen to be activated at the oxidation level of peroxide. From our results, we

cannot distinguish between an enzyme-bound peroxide ion and an enzyme-bound tetrahydropteridine hydroperoxide as the reactive oxygen intermediate in equation 4.

According to this scheme, the ratio of XH_4 oxidized to tyrosine formed would be determined by the relative rates of reactions 5a and 5b; these rates would, in turn, be dependent on the structure of both the substrate and the pteridine.

Our results rule out any mechanism for the hydroxylation reaction, such as that recently proposed for the analogous pteridine requiring tyrosine hydroxylase (Ikeda et al., 1966) in which the enzyme is first reduced by XH_4 followed by dissociation of XH_2 from the enzyme.

The results are also inconsistent with a considerable body of speculation about the mechanism of activation of molecular oxygen (Hamilton, 1966; Udenfriend, 1954).

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