THE EFFECT OF ENZYME CONCENTRATION, IONIC STRENGTH, AND TEMPERATURE ON THE STOICHIOMETRY OF THE PHENYLALANINE HYDROXYLASE REACTION

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Summary. When 7-methyltetrahydropterin is substituted for the natural cofactor, tetrahydrobiopterin, in the phenylalanine hydroxylase system, more than one mole of tetrahydropterin (or TPNH) is oxidized for each mole of tyrosine formed. Increasing the enzyme concentration, raising the ionic strength, and reducing the temperature of the reaction, lower the ratio of TPNH oxidation to tyrosine formation to nearly 1.0. A possible explanation for these results is that phenylalanine hydroxylase can exist in multiple forms that differ in the efficiency with which the oxidation of 7-methyltetrahydropterin is coupled to hydroxylation of substrate.

Phenylalanine hydroxylase catalyzes the conversion of phenylalanine to tyrosine according to reaction 1 (Kaufman, 1959), where XH_4 stands for tetrahydropterin[1] and XH_2 for quinonoid dihydropterin (Kaufman, 1964). The reduction of the dihydropterin back to the tetrahydro level is catalyzed by a separate enzyme, dihydropteridine reductase, as shown in reaction 2 (Kaufman, 1957).

$$\text{Phenylalanine} + XH_4 + O_2 \rightarrow \text{tyrosine} + XH_2 + H_2O \qquad (1)$$

$$XH_2 + \text{TPNH} + H^+ \rightarrow XH_4 + \text{TPN}^+ \qquad (2)$$

When the hydroxylation reaction is carried out in the presence of the natural pterin cofactor, tetrahydrobiopterin

[1] Pterin refers to derivatives of 2-amino-4-pteridone (Pfleiderer, 1964).

(Kaufman, 1963), or with the 6-methyl or 6,7-dimethylpterin, one mole of tetrahydropterin is oxidized for each mole of tyrosine formed (Kaufman, 1959; Storm and Kaufman, 1968).

Certain alterations in the structure of the substrate or the pterin cofactor result in the oxidation of more than one mole of TPNH (or XH_4) for each mole of tyrosine formed (Storm and Kaufman, 1968). We report here that changes in the concentration of phenylalanine hydroxylase, the ionic strength, and the temperature of the reaction also modify the ratio of TPNH oxidized to tyrosine formed. These results suggest that phenylalanine hydroxylase can exist in multiple forms of differing catalytic activity.

Materials and Methods

L-phenylalanine was obtained from Nutritional Biochemical Company. Crystalline catalase and TPNH were purchased from C. F. Boehringer and Soehne of Mannheim, Germany. 7-methylpterin (2-amino, 4-hydroxy, 7-methylpteridine) was prepared by a modification of standard methods[2]. The pterin was catalytically hydrogenated over PtO_2, lyophilized, and stored as the hydrochloride at -20°. The concentration of the tetrahydropterin was determined spectrophotometrically in 2 N perchloric acid. A molar extinction coefficient at 265 mμ of 1.86×10^4 was used for 7-methyltetrahydropterin[2]. This value is close to that reported for the 6-methyltetrahydropterin (Whitely and Huennekens, 1967). The phenylalanine hydroxylase and dihydropterin reductase were purified through the calcium phosphate gel step (Kaufman, 1962). Phenylalanine hydroxylase was further purified by the use of DEAE cellulose,

[2] Storm, C. B., Shiman, R., and Kaufman, S., manuscript in preparation.

Sephadex G-200, and Bio-gel A 0.5 m column chromatography (Kaufman, Methods in Enzymology, in press). Protein concentrations were determined spectrophotometrically (Warburg and Christian, 1941-42). Initial velocities of TPNH oxidation were determined by following absorbance at 340 mμ in a Gilford recording spectrophotometer. Constant temperature was maintained with a Lauda bath (Brinkmann) by the circulation of water through a jacket surrounding the cuvette chamber. Tyrosine formation was measured fluorometrically by the nitroso-naphthol method (Waalkes and Udenfriend, 1957).

Results and Discussion

When 7-methyltetrahydropterin was used in place of 6-methyltetrahydropterin or tetrahydrobiopterin there was more TPNH oxidized than tyrosine formed (Storm and Kaufman, 1968). Increasing the concentration of phenylalanine hydroxylase reduced the ratio of TPNH oxidized to tyrosine formed (Figure 1). This ratio was further reduced by increasing the ionic strength of the reaction mixture. Sulfate ions were as effective as phosphate ions in this respect. Ionic strength had the most pronounced influence on the stoichiometry of the reaction when the enzyme concentration was low. The enzyme concentration curve obtained at low ionic strength apparently extrapolates to ratios higher than 4.5. Under these conditions, the enzyme is catalyzing a reaction that is predominantly a phenylalanine-dependent oxidation of the tetrahydropterin. It is possible that at extremely low ionic strength and enzyme concentration, the only catalytic activity that would be manifest by phenylalanine hydroxylase, with the 7-methyltetrahydropterin as cofactor, would be that of a tetrahydropterin oxidase.

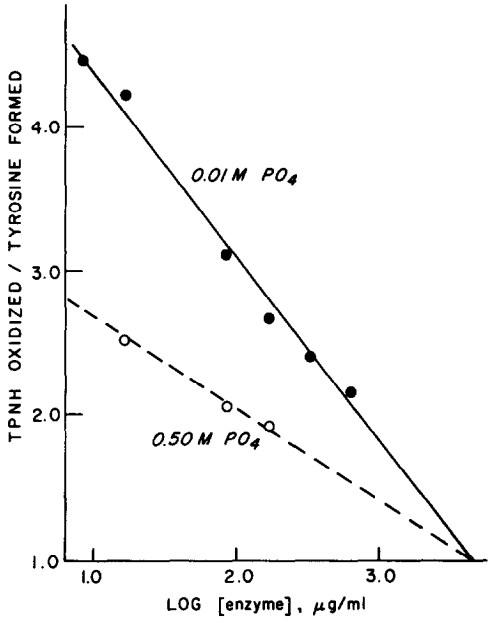

Figure 1. The effects of enzyme concentration and ionic strength on the stoichiometry of the phenylalanine hydroxylase reaction. The assay mixture contained potassium phosphate buffer, pH 6.3, and phenylalanine hydroxylase at the indicated concentrations. The other components in the assay mixture were phenylalanine, 2 mM, dihydropteridine reductase, 0.1 mg/ml, catalase, 0.04 mg/ml, TPNH, 0.2 mM, and 7-methyltetrahydropterin at 0.08 mM. The final volume was 1.0 ml and the temperature was 25°.

The effect of enzyme concentration on the reaction rate was evaluated from a plot of the logarithm of the initial rate of TPNH oxidation and tyrosine formation against the logarithm of the enzyme concentration (Figure 2). The slopes were calculated by the method of least squares. The slope of the line for TPNH oxidation is nearly 1.0 (0.98), an indication that the rate of TPNH oxidation is a linear function of enzyme concentration. Thus, the rate of 7-methyltetrahydropterin (or TPNH) oxidation per mg of enzyme is constant throughout this enzyme concentration range. However, the slope of the line for tyrosine formation is greater than 1.0 (1.13). Therefore,

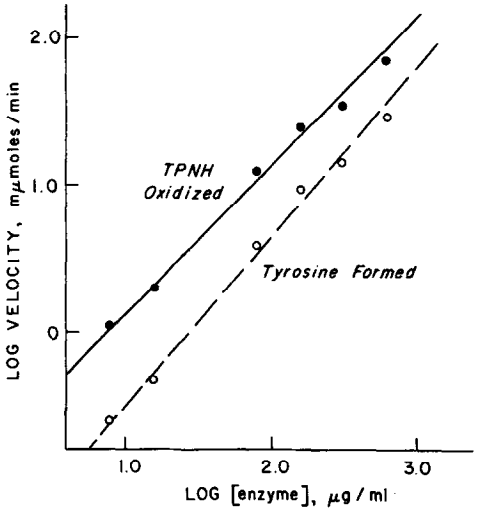

Figure 2. The initial velocities of TPNH oxidation and tyrosine formation as a function of phenylalanine hydroxylase concentration. The assay conditions were the same as those indicated for Fig. 1. Concentration of phosphate buffer, pH 6.3, was 0.01 M. The initial velocity of TPNH oxidation was measured directly as the decrease in absorbance at 340 mμ. The initial velocity of tyrosine formation was calculated by dividing the initial velocity of TPNH oxidation by the TPNH-to-tyrosine ratio as determined at the end of the reaction. This is a valid calculation because under any given set of conditions the TPNH-to-tyrosine ratio remains constant during the course of the reaction.

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the rate of tyrosine formation per mg of enzyme decreases with decreasing enzyme concentration.

The effect of temperature on the stoichiometry of the reaction was assessed. Elevation of the reaction temperature increased the TPNH-to-tyrosine ratio in a stepwise manner (Figure 3). As was the case with ionic strength, the temperature change had a more pronounced effect at low enzyme concentrations.

Our previous studies demonstrated that alterations in the structures of the pterin cofactor and of the substrate could markedly affect the stoichiometry of the hydroxylation

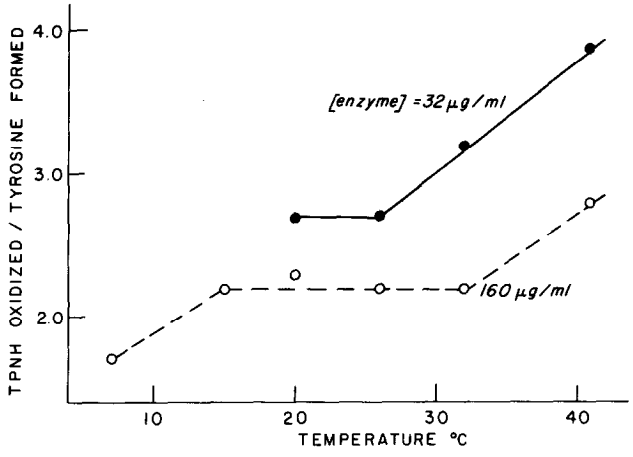

Figure 3. The effect of temperature on the stoichiometry of the phenylalanine hydroxylase reaction. The potassium phosphate buffer, pH 6.3, was present at 0.005 M. The concentration of TPNH was 0.1 mM and of 7-methyltetrahydropterin was 0.04 mM. All other components of the assay were present in the same concentration as described for Fig. 1.

reaction (Storm and Kaufman, 1968). The present studies show that enzyme concentration, ionic strength and temperature are also important factors in determining the stoichiometry.

The present results, in particular the variation in the ratio of TPNH oxidized to tyrosine formed with enzyme concentration, suggest that phenylalanine hydroxylase from rat liver may undergo reversible association. According to this idea, in the presence of the 7-methyltetrahydropterin, the higher polymer would catalyze a reaction in which tetrahydropterin oxidation is efficiently coupled to hydroxylation of phenylalanine, leading to a TPNH/tyrosine ratio that approaches 1.0. On the other hand, with the lower polymer, the oxidation of the tetrahydropterin would be inefficiently coupled to hydroxylation, leading to high TPNH/tyrosine ratios.

As far as we are aware, this is the first example of an enzyme system where the stoichiometry of the reaction catalyzed

varies with the concentration of the enzyme.

Work is in progress to determine if the factors that influence the stoichiometry affect the degree of polymerization of phenylalanine hydroxylase.

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