Kinetics of Tetrahydrobiopterin Synthesis by Rabbit Brain Dihydrofolate Reductase

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Product identification and kinetic data are presented for the conversion of 7,8-dihydrobiopterin into tetrahydrobiopterin by purified rabbit brain dihydrofolate reductase.

Rabbit brain contains dihydrofolate reductase (5,6,7,8-tetrahydrofolate-NADP⁺ oxidoreductase, EC 1.5.1.3), which has been characterized after partial purification (Spector *et al.*, 1977). In purified form, the brain enzyme reduces both dihydrofolate to tetrahydrofolate and 7,8-dihydrobiopterin to tetrahydrobiopterin (Spector *et al.*, 1978). The latter reaction probably provides the final step in brain for the formation *de novo* of tetrahydrobiopterin from GTP (Gal & Sherman, 1976; Gal *et al.*, 1976, 1977; Eto *et al.*, 1976; Spector *et al.*, 1978). Tetrahydrobiopterin is a cofactor for phenylalanine hydroxylase, tyrosine hydroxylase and tryptophan hydroxylase (Friedman *et al.*, 1972; Kaufman, 1973).

The present paper reports the kinetics of 7,8-dihydrobiopterin conversion into tetrahydrobiopterin as well as confirmation that the tetrahydrobiopterin formed is, in fact, a functional cofactor for the phenylalanine hydroxylase system.

Materials and Methods

All materials were obtained as stated previously (Spector *et al.*, 1977, 1978). Rabbit brain dihydrofolate reductase was prepared and purified to homogeneity as previously described (Spector *et al.*, 1977, 1978). 7,8-Dihydrobiopterin was generated from the naturally occurring L-erythro stereoisomer of biopterin (Kaufman, 1967).

To study the kinetics of 7,8-dihydrobiopterin conversion into tetrahydrobiopterin by purified rabbit brain dihydrofolate reductase reaction mixtures containing 100 μ mol of potassium phosphate buffer, pH 6.8, 5 μ mol of 2-mercaptoethanol, 0.05 μ mol of NADPH, 6 μ g of purified brain dihydrofolate reductase and various amounts of 7,8-dihydrobiopterin were prepared in a volume of 1.0 ml. The disappearance of NADPH at 25°C was monitored continuously at 340 nm (Mathews *et al.*, 1963). The initial rate of the reaction was proportional to the amount of enzyme added up to 8 μ g.

To show that tetrahydrobiopterin synthesized enzymically from 7,8-dihydrobiopterin by purified brain dihydrofolate reductase could act as a cofactor for phenylalanine hydroxylase, the activity of phenylalanine hydroxylase in the presence of the synthesized cofactor was measured. Phenylalanine hydroxylase was prepared as previously described (Kaufman & Fisher, 1970). The tetrahydrobiopterin was generated at 37°C for 30 min in a reaction mixture containing 40 μ mol of sodium phosphate buffer, pH 6.8, 1.2 μ mol of 2-mercaptoethanol, 3.3 μ mol of sodium ascorbate, 0.2 μ mol of NADPH, 0.3 μ mol of 7,8-dihydrobiopterin and 4 μ g of purified brain dihydrofolate reductase in 1.0 ml. From this incubation, 5 nmol of tetrahydrobiopterin was added to a phenylalanine hydroxylase assay mixture containing 100 μ mol of potassium phosphate buffer, pH 6.8, 1 μ mol of phenylalanine, 10 μ mol of Cleland's reagent (dithiothreitol) and 1.2 mg of liver phenylalanine hydroxylase in 1.0 ml. The reaction was monitored continuously at 275 nm against a blank that lacked phenylalanine (Miller *et al.*, 1975). No conversion of phenylalanine into tyrosine occurred when bovine serum albumin was substituted for phenylalanine hydroxylase.

Results and Discussion

Fig. 1 shows the kinetics of 7,8-dihydrobiopterin reduction by purified brain dihydrofolate reductase. The K_m is 17 μ M and the V_{max} is 0.74 μ mol/min per mg under these conditions. The respective values for dihydrofolate reduction by this enzyme are 0.9 μ M and 7.9 μ mol/min per mg when 50 mM-Tris/maleate buffer, pH 7.5, was substituted for potassium phosphate buffer (Spector *et al.*, 1978).

Column-chromatographic techniques were previously utilized to identify tetrahydrobiopterin as the reaction product of 7,8-dihydrobiopterin and NADPH in the presence of purified brain dihydro-

Fig. 1. Double-reciprocal plot to determine K_m (7,8-dihydrobiopterin) of dihydrofolate reductase from normal rabbit brain

A straight line was drawn by the method of least squares.

Table 1. Requirements of phenylalanine hydroxylase assay

The concentrations of the components are given in the Materials and Methods section. Assays were performed on a Beckman model 25 spectrophotometer with 0.1 A_{275} unit full-scale deflection for all rate determinations. The initial part of the reaction progress curve was always linear (about 0.01 A_{275} unit/min). Phenylalanine hydroxylase was purified from rat liver to the second $(\text{NH}_4)_2\text{SO}_4$ precipitation stage as previously described (Kaufman & Fisher, 1970). The preparation contained neither dihydropteridine reductase nor dihydrofolate reductase activity. The components of the mixture (i.e. 2-mercaptoethanol, ascorbate, NADPH and 7,8-dihydrobiopterin) generating tetrahydrobiopterin via purified brain dihydrofolate reductase have no effect on the phenylalanine hydroxylase assay.

Components	Reaction rate (nmol/min)	Percentage of that for complete reaction
Complete reaction	7.8	100
Without phenylalanine	0	0
Without tetrahydrobiopterin mixture	0	0
Without Cleland's reagent	6.4	82

folate reductase (Spector *et al.*, 1978). Table 1 shows that 7,8-dihydrobiopterin is reduced to functionally active tetrahydrobiopterin by brain dihydrofolate reductase since the tetrahydrobiopterin serves as cofactor for phenylalanine hydroxylase (Kaufman, 1967). The complete reaction mixture converts phenylalanine into tyrosine at 7.8 nmol/min per mg. There is no re-

action without the addition of exogenous tetrahydrobiopterin. Cleland's reagent may be substituted for dihydropteridine reductase since it can continuously recycle tetrahydrobiopterin by instantaneously reducing quinonoid dihydrobiopterin (Miller *et al.*, 1975). Leaving out Cleland's reagent did not significantly alter the initial rate of tyrosine formation (Table 1); however, the reaction is then limited by the amount of added tetrahydrobiopterin.

These data extend previous chromatographic studies (Spector *et al.*, 1978) by utilizing tetrahydrobiopterin in a functional assay (Kaufman, 1967). Tetrahydrobiopterin is thereby unequivocally established as the product of the brain dihydrofolate reductase-catalysed reaction between L-erythro-dihydrobiopterin and NADPH. Also, the proposed last step in the synthesis *de novo* of tetrahydrobiopterin from GTP in brain becomes more firmly established (Gal *et al.*, 1977; Eto *et al.*, 1976; Spector *et al.*, 1978).

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