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7-Tetrahydrobiopterin is an uncoupled cofactor for rat hepatic phenylalanine hydroxylase

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Rat hepatic phenylalanine hydroxylase requires both a tetrahydropterin cofactor and molecular oxygen to convert phenylalanine to tyrosine. During the physiological hydroxylation, a single mol of the natural cofactor, tetrahydrobiopterin, is oxidized for each mol of phenylalanine converted to tyrosine. Artificial conditions have been devised in which the oxidation of the tetrahydropterin is uncoupled from the hydroxylation of the aromatic amino acid substrate. Recently, an isomer of tetrahydrobiopterin, 7-tetrahydrobiopterin, has been isolated from the urine of certain mildly hyperphenylalaninemic children. We report in this communication that 7-tetrahydrobiopterin may be an inefficient cofactor for phenylalanine hydroxylase because, in vitro, the phenylalanine-dependent oxidation of 7-tetrahydrobiopterin is accompanied by the hydroxylation of the aromatic amino acid substrate only about 15% of the time, i.e. the enzymatic oxidation of 7-tetrahydrobiopterin is about 85% uncoupled from the hydroxylation of the amino acid substrate.

Tetrahydrobiopterin; 7-Tetrahydrobiopterin; Phenylalanine hydroxylase; Uncoupled oxidation; Phenylketonuria

1. INTRODUCTION

Phenylalanine hydroxylase shows an absolute requirement for a reduced pterin cofactor that can be met by the naturally-occurring coenzyme, tetrahydrobiopterin, BH_4[1] [1] or by several synthetic analogs of BH_4 [2,3]. As shown in equation (1), in the presence of molecular oxygen, this monooxygenase catalyzes the hydroxylation of phenylalanine to tyrosine with the concomitant oxidation of BH_4 to the 4a-carbinolamine, 4a-hydroxytetrahydrobiopterin [4]. 4a-Carbinolamine (BH_3OH) can then be dehydrated to quinonoid dihydrobiopterin (qBH_2) and water by 4a-carbinolamine dehydratase, equation (2) [4–6]. A third enzyme, dihydropteridine reductase, catalyzes the NADH-dependent reduction of qBH_2 back to BH_4, equation (3) [7]. Thus, in this multi-enzyme system, BH_4 functions catalytically as phenylalanine, molecular oxygen and NADH are converted to tyrosine, water, and NAD^+.

$$Phe + O_2 + BH_4 \longrightarrow Tyr + BH_3OH \quad (1)$$

$$BH_3OH \longrightarrow qBH_2 + H_2O \quad (2)$$

$$qBH_2 + NADH + H^+ \longrightarrow BH_4 + NAD^+ \quad (3)$$

Abbreviations: BH_4, 6-[dihydroxypropyl-(L-*erythro*)-5,6,7,8-tetrahydropterin] (tetrahydrobiopterin); 7-BH_4, 7-[dihydroxypropyl-(L-*erythro*)-5,6,7,8-tetrahydropterin]; 6MPH_4, 6-methyl-5,6,7,8-tetrahydropterin; BH_3OH, 4a-hydroxytetrahydrobiopterin (4a-carbinolamine)

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The absence of the enzymes involved in either the biosynthesis or the regeneration of BH_4 can result in a severe form of hyperphenylalaninemia [8]. Recently, a group of mildly hyperphenylalaninemic patients have been identified who excrete the unusual pterin, 7-substituted biopterin, along with the normal 6-substituted pterin in their urine [9,10]. The formation of this novel pterin may result from the absence of 4a-carbinolamine dehydratase [11,12]. The pathogenic role, if any, of 7-tetrahydrobiopterin (7-BH_4) in the resulting hyperphenylalaninemia is still unclear.

As outlined above, the stoichiometry for the BH_4-dependent hydroxylation of phenylalanine to tyrosine by phenylalanine hydroxylase is one mol of tyrosine formed per mol of BH_4 oxidized [7,13]. This stoichiometry can be altered in vitro by the use of certain analogs of the pterin cofactor and/or phenylalanine [14–18]. In all of these cases the amount of cofactor oxidized exceeds the amount of the aromatic amino acid hydroxylated, i.e. the tetrahydropterin oxidation is partially uncoupled from the hydroxylation of the substrate [13]. We now report that under conditions in which the oxidation of the natural cofactor by phenylalanine hydroxylase is completely coupled, the analogous reaction with 7-BH_4 is approximately 85% uncoupled. These results strongly suggest that 7-BH_4 is an inefficient cofactor in vivo.

2. MATERIALS AND METHODS

L-Phenylalanine, L-tyrosine, and NADH were purchased from Sigma. Hydrogen peroxide was obtained from Fisher Scientific Co. Beef liver catalase and bovine erythrocyte superoxide dismutase were

purchased from Boehringer Mannheim Biochemicals. (6*R*)-BH_4, (6*R*,*S*)-BH_4, (6*R*,*S*)methyltetrahydropterin ((6*R*,*S*)MPH_4), and (7*R*,*S*)-BH_4 were purchased from Dr. B. Schirks' Laboratory, Jona, Switzerland. Phenylalanine hydroxylase was purified from rat liver by a combination of 2 methods [19,20] in what has been described as the hybrid procedure [21]. The pure enzyme was between 40–60% active [22,23]. Sheep liver dihydropteridine reductase was purified by the method of Crane et al. [24]. All other reagents were of the highest quality available.

The oxidation of NADH was monitored at 340 nm with the use of a Gilford 250 spectrophotometer that was temperature-controlled by a Lauda K-2/R bath. An extinction coefficient of 6220 $M^{-1} \cdot cm^{-1}$ was used to calculate the amount of NADH consumed. All measurements were corrected for the contribution due to the autooxidation of the tetrahydropterins. All reactions were started upon the addition of the tetrahydropterin following a 3 min, 25°C preincubation of phenylalanine hydroxylase with phenylalanine. The formation of tyrosine was determined colorimetrically [25]. Hydrogen peroxide was measured electrochemically with the model 27 Industrial Analyzer, purchased from Yellow Springs Instruments Co. Inc., with a Linear 1200 recorder. An extinction coefficient of 43.6 $M^{-1} \cdot cm^{-1}$ at 240 nm was used to calibrate the standard solutions of the hydrogen peroxide [26]. The volume of the reaction chamber of the Industrial Analyzer was 0.46 ml.

3. RESULTS AND DISCUSSION

As described in section 1, the conversion of phenylalanine to tyrosine by phenylalanine hydroxylase involves the oxidation of a tetrahydropterin cofactor whose product is, in turn, reduced by the NADH-dependent dihydropteridine reductase [13]. Thus, in the presence of dihydropteridine reductase, the phenylalanine-dependent oxidation of tetrahydropterins can be monitored indirectly by the linked oxidation of NADH. With the natural cofactor (6*R*)-BH_4 [1,27–30], the amount of NADH oxidized is equivalent to the quantity of tyrosine formed (Table I); this stoichiometry is termed fully-coupled. As can be seen in Table I, this relationship still holds true for the reaction with the synthetic mixture of the *R* and *S* diasteriomers, suggesting that the stereochemistry of the cofactor has no effect on the stoichiometry of the oxidation. Similarly, the replacement of (6*R*,*S*)-BH_4 with the racemic mixture of $6MPH_4$, a synthetic cofactor analog [2], is also characterized by a 1:1 ratio between the amount of NADH oxidized and tyrosine formed. In contrast, this ratio increases dramatically in the presence of (7*R*,*S*)-BH_4 which is consistent with (7*R*,*S*)-BH_4 being a partially uncoupled cofactor.

The oxidation of both tetrahydropterins is nonlinear (Fig. 1A) and, at least under these experimental conditions, it is apparent that the reaction proceeds more quickly with the natural cofactor. Although it is not so clear from these data, the rate of the reaction also falls off more rapidly with (7*R*,*S*)-BH_4 (not shown). Inactivation of phenylalanine hydroxylase during the course of the reaction is believed to be the major cause of this nonlinearity [31]. Hydrogen peroxide, a product of the autooxidation of tetrahydropterins, is one of the main contributors to this inactivation of phenylalanine hydroxylase [15]. As will be described below, the apparent decrease in stability of phenylalanine hydroxylase in the presence of (7*R*,*S*)-BH_4, but in the absence of catalase, is consistent with the reaction being at least partially uncoupled.

The uncoupled oxidation of tetrahydropterins by phenylalanine hydroxylase has been studied extensively [14–18,32,33]. There is no net hydroxylation of the aromatic amino acid substrate during the uncoupled portion of the reaction and a new product, hydrogen peroxide is observed [15,16]. After the blank due to the autooxidation of the tetrahydropterins is subtracted, no hydrogen peroxide is produced in the fully-coupled oxidation of (6*R*)-BH_4, whereas a significant amount of hydrogen peroxide is formed when the natural cofactor is replaced by (7*R*,*S*)-BH_4 (Fig. 1B). These results are consistent with the oxidation of (7*R*,*S*)-BH_4 by phenylalanine hydroxylase being partially uncoupled from the hydroxylation of the substrate.

Table I

Stoichiometry of the phenylalanine-dependent oxidation of tetrahydropterins catalyzed by phenylalanine hydroxylase

Cofactor	TYR (nmol)	NADH (nmol)	NADH/TYR	% Coupled
6(*R*)-BH_4	48,42	43,43	0.96	100
6(*R*,*S*)-BH_4	48,41	47,42	1.0	100
6(*R*,*S*)-MPH_4	40,42	43,43	1.0	0.95
7(*R*,*S*)-BH_4	7.5,9.5	45,57.4	6.0	0.17

Phenylalanine hydroxylase (63 nM) was incubated for 3–5 min at 25°C with 0.3 mM phenylalanine in a reaction mixture containing 0.1 M potassium phosphate, pH 6.8, 0.1 mg/ml catalase, 0.13 mM NADH, and an excess of dihydropteridine reductase. The reaction was started by the addition of 0.1 mM tetrahydropterin. The phenylalanine-dependent oxidation of NADH was monitored at 340 nm and allowed to proceed until a decline of about 0.3–0.4 absorbance units was observed. The reaction was then stopped by the addition of 7.5% trichloroacetic acid. The amount of NADH oxidized and tyrosine produced was quantified as described in section 2. It should be noted that because of differences in the cofactor activities of the tetrahydropterins used, the incubation time required to achieve similar decreases in absorbance was not the same for each pterin; the listed values are the amounts of products formed rather than the rates of formation.

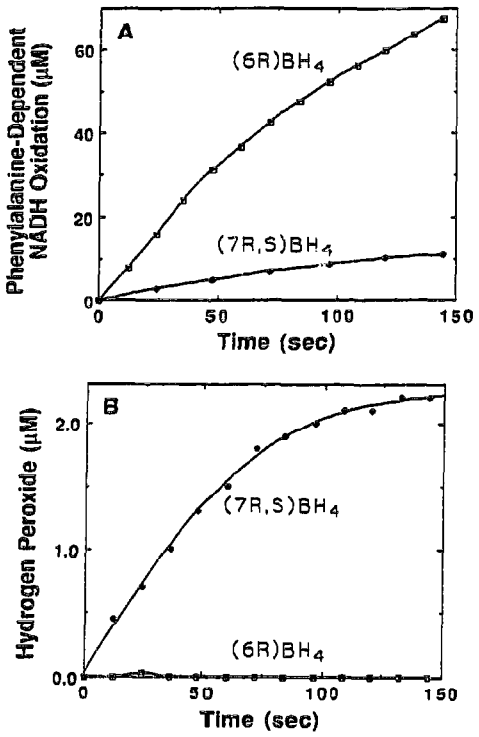

Fig. 1. The phenylalanine-dependent oxidation of (6*R*)-BH_4, and (7*R*,*S*)-BH_4 by phenylalanine hydroxylase. (A) The oxidation of (6*R*)-BH_4, open squares, and (7*R*,*S*)-BH_4, closed squares, was monitored for the linked oxidation of NADH by dihydropteridine reductase. The reaction was performed at 27°C, in 0.09 M potassium phosphate, pH 6.8, that contained 45 mM potassium chloride, 40 μg/ml superoxide dismutase, 0.13 mM NADH, 0.31 mM phenylalanine, 95 nM phenylalanine hydroxylase, an excess of dihydropteridine reductase and 30 μM tetrahydropterin as described in section 2. (B) The oxidation of (6*R*)-BH_4, open squares, and (7*R*,*S*)-BH_4, closed squares, was monitored for the liberation of hydrogen peroxide as described in section 2. The reaction was performed at 27°C, in 0.9 M potassium phosphate pH 6.8 that contained 45 mM potassium chloride, 50 μg/ml superoxide dismutase, 0.11 mM NADH, 0.27 mM phenylalanine, 82 nM phenylalanine hydroxylase, an excess of dihydropteridine reductase and 27 μM tetrahydropterin.

Although the kinetic trace for the formation of hydrogen peroxide during the oxidation (7*R*,*S*)-BH_4 (Fig. 1B) appears to follow that for the corresponding oxidation of NADH (Fig. 1A), there is clearly more NADH oxidized than hydrogen peroxide produced. If we estimate the amounts of hydrogen peroxide formed and NADH consumed during the most linear portion of the reaction, i.e. 0–1.0 min, and adjust this figure by subtracting the amount of tetrahydropterin oxidixed during the coupled portion of the reaction, i.e. 15% (Table I), then the percentage of hydrogen peroxide formed relative to (7*R*,*S*)-BH_4 oxidized during the uncoupled part of the reaction is about 30%. This value is in good agreement with those estimated for other uncoupled oxidations of tetrahydropterins by phenylalanine hydroxylase [34].

The phenylalanine-dependent oxidation of the artificial cofactor, (7*R*,*S*)-methyltetrahydropterin, by phenylalanine hydroxylase has long been known to be partially uncoupled [16] and thus it is not surprising that 7-BH_4 reacts in an analogous manner. On the other hand, 7-BH_4 is the first naturally-occurring cofactor to display this property when the hydroxylation of the natural substrate, phenylalanine, is examined. The mild hyperphenylalaninemia that is associated with the excretion of increased amounts of 7-BH_4 is consistent with our findings because the oxidation of (7*R*,*S*)-BH_4 by phenylalanine hydroxylase is accompanied by phenylalanine hydroxylation only about 15% of the time. Further studies are necessary, however, to ascertain the effect, if any, of this putative inefficiency of 7-BH_4 utilization in vivo.

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