Phenylalanine hydroxylase stimulator protein is a 4α-carbinolamine dehydratase*

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Phenylalanine hydroxylase stimulator protein is shown to be an enzyme that catalyzes the dehydration of the 4α-carbinolamine, derived from the turnover of 6-methyltetrahydropterin with phenylalanine hydroxylase, to form p-quinonoid-6-methyltetrahydropterin. This conclusion is based on the facts that: 1) a linear dependence on phenylalanine hydroxylase stimulator concentration of the first order rate of decay at low levels of 4α-carbinolamine is observed; 2) the physical separation of phenylalanine hydroxylase from phenylalanine hydroxylase stimulator and 4α-carbinolamine gives identical kinetic parameters with those obtained in its presence, thus indicating no catalytically significant protein-protein interaction; and 3) the 4α-carbinolamine analog, 5-deaza-4α-hydroxy-6-methyltetrahydropterin is an inhibitor of phenylalanine hydroxylase stimulator activity.

The structure of the tetrahydropertin-derived intermediate formed by phenylalanine hydroxylase (EC 1.14.16.1) during catalysis has recently been shown to be the 4α-hydroxy adduct based on 13C NMR and UV data (1, 2). The 4α-CA† can dehydrate nonenzymatically to form the p-quinonoid dihydropterin (3), which is recycled to the tetrahydropterin by NADH and dihydropteridine reductase (EC 1.6.99.7). The abbreviations used are: 4α-CA, 4α-carbinolamine of 6-methyltetrahydropterin; PHS, phenylalanine hydroxylase stimulator; 6-MPH2, 6-methyldihydropterin.

PAH, phenylalanine hydroxylase; DHPR, dihydropteridine reductase.

Phenylalanine hydroxylase stimulator protein, which has been purified and studied (4-6), was initially thought to influence the association-dissociation of phenylalanine hydroxylase (4). Subsequently, however, it was shown that PHS catalyzes the conversion to quinonoid dihydropterin of a pterin intermediate in the phenylalanine hydroxylase reaction (7). The pterin intermediate was postulated to be the 4α-carbinolamine derived from the corresponding tetrahydropterin (7), a postulate that was later shown to be correct (1, 2). The purpose of this paper is to present unequivocal evidence that PHS is a 4α-carbinolamine dehydratase.

EXPERIMENTAL PROCEDURES

Materials—Phenylalanine hydroxylase and PHS, purified from rat liver as previously described (4, 5), were pure as judged by sodium dodecyl sulfate-gel electrophoresis on 12% and 15% acrylamide slab gels, respectively, according to the method of Laemmli (8). There was no cross-contamination of the activities in either protein. 6-MPH2, (2), trans-5-deaza-4α-hydroxy-6-methyltetrahydropterin (10), and 6-phenyl-5-thiatetrahydropterin (11) were synthesized as previously described. Catalase was purchased from Sigma.

Methods—UV spectra were obtained on either a Cary 118 or Cary 219 spectrophotometer. Phenylalanine hydroxylase was assayed by the method of Shiman et al. (8). PHS was assayed by measuring the increase in the first order rate of decay of 4α-CA to p-quinonoid-6-MPH2 at 244 nm. Assay conditions were as follows. A cuvette containing 0.1 M Tris-HCl, pH 8.45, 1 mM L-phenylalanine, and 45 pg of PHS (in 10 mM Tris-HCl, pH 7.0, 0.15 M KCl) was added at t = 0 s. The concentration of PHS was monitored. The first order rate constant (kobs) was determined from the slope of a plot of ln [Aα - Aβ] versus t. Extinction coefficients at 244 nm (an isosbestic point for quinonoid- and 7,8-6-MPH2) for 6-MPH, 4α-CA, and p-quinonoid-6-MPH2 are 4,000, 16,500, and 7,200 M⁻¹ cm⁻¹, respectively. From the observed kobs of the reaction progress curve the concentrations of the three species were calculated as described by Harris (12).

Inhibition studies using 5-deaza-4α-hydroxy-6-MPH2 were carried out by adding equivalent amounts to blank and sample cuvettes containing 0.1 M Tris-HCl pH 8.45, 1 mM L-phenylalanine, and 72 µg of phenylalanine hydroxylase, both with and without 50 µg of catalase, at time zero, followed by addition of 12 µM 6-MPH2 at t = 90 s and 3 µg of PHS at t = 130 s. The amount of inhibitor present at t = 150 s, where kobs was determined, was calculated to be 73% of the initial concentration due to the instability of 5-deaza-4α-hydroxy-6-MPH2 (k = 0.0021 s⁻¹) under these conditions. The amount of 4α-CA at 150 s was estimated to be between 1.7 and 5.5 µM depending upon inhibitor concentration. The product of the decomposition of 5-deaza-4α-hydroxy-6-MPH2 (10) does not inhibit PHS.

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* This investigation was supported by National Science Foundation Grant PCM 81-03670. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: 4α-CA, 4α-carbinolamine of 6-methyltetrahydropterin; PHS, phenylalanine hydroxylase stimulator; 6-MPH2, 6-methyltetrahydropterin; 6-MPH, 6-methyldihydropterin.

2%0 trans; 10% cis by 1H NMR.

3 The specific activity of phenylalanine hydroxylase in 0.1 M Tris, pH 8.45, is two-thirds that in 0.1 M KPi, pH 6.8 (8).
In some experiments physical separation of phenylalanine hydroxylase from the reaction mixture was accomplished by filtration via a 1-ml syringe through a Millex-PF (0.8 μm) cellulose ester membrane (Millipore). No phenylalanine hydroxylase activity is observed after filtration. At time zero, 11.8 μM 6-MPH₄ was added to 0.1 M Tris-HCl, pH 8.45, 1 mM L-phenylalanine and 57 μg of phenylalanine hydroxylase in 1-ml assay. After 60 s the mixture was filtered and at 80 s, 2 μg of PHS/ml were added and ¹⁴C₄ monitored.

RESULTS

The effect of PHS is most evident in the reaction progress curve of 6-MPH₄ with phenylalanine hydroxylase at pH 8.45, where no intermediate is observed in its presence (Fig. 1C).⁴ If PHS is added after the 4a-CA is generated by phenylalanine hydroxylase (see "Methods") an increase in the first order rate of decay (kₚ₅₈) of 4a-CA is observed (Fig. 1B). These results with the 4a-CA derived from 6-MPH₄ are in agreement with those previously reported on the effect of PHS on the 4a-CA derived from tetrahydrobiopterin (7). Assuming that the 4a-CA concentration is below its Kₘ for PHS and 6-MPH₄, kₚ₅₈ is the first order nonenzymatic rate of decay (= 0.010 s⁻¹) and kₚ₅₈ = (kₕₒ₅₈[P₅₈]/Kₘₕₙₐₕₐ) for PHS. Catalase has no effect on kₕₒ₅₈.

As can be seen from Fig. 2 there is a strict dependence of kₕₙₐₕₐ on PHS concentration. That PHS is acting as an enzyme to catalyze dehydration of 4a-CA and not in conjunction with phenylalanine hydroxylase is clearly demonstrated by the fact that the physical separation of phenylalanine hydroxylase from 4a-CA by filtration through a Millex-PF membrane and subsequent addition of PHS (3 μg/ml) gives an identical rate (kₕₒ₅₈ = 0.56 s⁻¹) with that observed in the presence of phenylalanine hydroxylase. In addition there is only a very slight effect (~7%) on kₕₒ₅₈ upon filtration of phenylalanine hydroxylase from the reaction mixture.

Further evidence consistent with the 4a-carbinolamine dehydratase activity of PHS is derived from the observation that 5-deaza-4a-hydroxy-6-MPH₄ is an inhibitor of the PHS reaction. Assuming that 4a-CA is below the Kₘ for PHS (see "Methods") and that inhibition of the deaza analog is competitive against 4a-CA, then:

\[ \frac{1}{k_{ₚ₅₈}} = \frac{K_{ₘₜ₈}}{V_{ₘₜ₈}} + \frac{K_I}{V_{ₘₜ₈}K_I} \]

Therefore a plot of 1/kₚ₅₈ versus I should be linear with Kᵢ = intercept/slope. The data in Fig. 3 give a value for Kᵢ = 48 μM. No inhibition is observed by 5-deza-4a-hydroxy-6-MPH₄ (100 μM) when assayed with phenylalanine hydroxylase and 6-MPH₄ (12 μM) either at pH 6.8 or pH 8.45. Also, there is no inhibition by 6-phenyl-5-thiatetrahydropterin (100 μM), a potent competitive inhibitor of phenylalanine hydroxylase (Kᵢ = 0.2 μM (11)), when assayed with PHS and 6-MPH₄ (12 μM).

DISCUSSION

The evidence that PHS is a 4a-carbinolamine dehydratase is based on the facts that: 1) a linear dependence on PHS concentration of the first order rate of decay at low concentrations of 4a-CA is observed; 2) there is no catalytically significant protein-protein interaction between phenylalanine hydroxylase and PHS; and 3) the 5-deaza-4a-hydroxy analog is an inhibitor of PHS activity. It has now been demonstrated that both tetrahydrobiopterin (7) and 6-MPH₄ (1) form a 4a-carbinolamine during phenylalanine hydroxylase turnover which is more stable at higher pH values. The relative stability of 4a-CA at higher pH values is not surprising since carbinolamine dehydrations are acid-catalyzed reactions (13). Since the 4a-CA derived from both tetrahydropterins serves as a substrate for PHS, the side chain at C-6 is not involved in the chemical mechanism of the dehydration.

It is interesting to note that inhibitors of phenylalanine hydroxylase are not detectible inhibitors for PHS and vice versa even though the product of phenylalanine hydroxylase

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⁴ The absence of 4a-CA in curve C suggests that PHS utilizes 4a-CA with either the R or S configuration at C-6.

⁵ For example, at t = 75 s, 6-MPH₄ ≈ 1.1 μM; 4a-CA = 8.5 μM; quinonoid-6-MPH₄ = 5.4 μM for curve A in Fig. 1.
is the substrate for PHS. This implies that the equilibrium phenylalanine hydroxylase \( 4a-\text{CA} \rightleftharpoons \text{phenylalanine hydroxylase + 4A-CA} \) favors dissociation and that \( 4a-\text{CA} \) is not a strong product inhibitor. Consequently, PHS stimulation of phenylalanine hydroxylase turnover should be viewed as maintenance at higher steady state levels of the tetrahydropterin pool during the recycling assay (6).

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J. Biol. Chem. 1983, 258:10960-10962.

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