Catalytic Characterization of 4a-Hydroxytetrahydropterin Dehydratase†

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ABSTRACT: The cofactor product of the aromatic amino acid hydroxylases, 4a-hydroxy-6(*R*)-tetrahydrobiopterin, requires dehydration before tetrahydrobiopterin can be regenerated by dihydropteridine reductase. Carbinolamine dehydration occurs nonenzymatically, but the reaction is also catalyzed by 4a-hydroxytetrahydropterin dehydratase. This enzyme has the identical amino acid sequence to DCoH, the dimerization cofactor of the transcription regulator, HNF-1α. The catalytic activity of rat liver dehydratase was characterized using a new assay employing chemically synthesized 4a-hydroxytetrahydropterins. The enzyme shows little sensitivity to the structure or configuration of the 6-substituent of its substrate, with K_m's for 6(*S*)-methyl, 6(*R*)-methyl, 6(*S*)-propyl, and 6(*R*)-L-*erythro*-dihydroxypropyl all between 1.5 and 6 μM. Turnover numbers at 37 °C are 50−90 s^{-1} at pH 7.4 and 2.5−3-fold lower at pH 8.4. Both 4a(*R*)- and 4a(*S*)-hydroxytetrahydropterins are good substrates. The quinoid dihydropterin products are strong inhibitors of the dehydratase with K_I's about one half of their respective K_m's, but no inhibition was observed with 7,8-dihydropterins or tetrahydropterins. The enzyme contains no metals and no phosphorus. Reaction mechanisms which involve either acid and/or base catalysis are discussed. 4a-Hydroxy-6(*R*)-tetrahydrobiopterin was determined not to be a product inhibitor of phenylalanine hydroxylase. It is concluded that the dehydratase (which was found to be 6 μM in rat liver) is essential *in vivo* to prevent rearrangement of 4a-hydroxy-6(*R*)-tetrahydrobiopterin and to maintain the supply of tetrahydrobiopterin cofactor for the hydroxylases under conditions where the nonenzymatic rate would be inadequate.

The aromatic amino acid hydroxylases each require the reduced pteridine cofactor tetrahydrobiopterin to activate molecular oxygen and hydroxylate the aromatic ring of the amino acid substrate. The cofactor is then released from the enzyme as 4a-hydroxytetrahydrobiopterin which must be dehydrated to quinoid dihydrobiopterin before it can be reduced by the NADH-dependent dihydropteridine reductase to regenerate tetrahydrobiopterin. Dehydration of 4a-hydroxytetrahydropterin to quinoid dihydrobiopterin is catalyzed by the enzyme 4a-hydroxytetrahydropterin dehydratase (Scheme 1). The enzyme which catalyzes reduction of quinoid dihydropterin to tetrahydropterin has been well-characterized (Whiteley et al., 1993), but little is known about the dehydratase. While it has been shown that this protein catalyzes dehydration of 4a-hydroxytetrahydropterins (Huang & Kaufman, 1973; Lazarus et al., 1983), the reaction also occurs spontaneously. Under most *in vitro* assay conditions of the hydroxylases, the nonenzymatic rate of dehydration of 4a-hydroxytetrahydropterin is sufficiently rapid that the dehydratase is not required.

4a-Hydroxytetrahydropterin dehydratase is a tetramer of identical subunits with molecular mass of 12 000 Da (Huang et al., 1973). It has recently been reported that 4a-hydroxytetrahydropterin dehydratase has the same amino acid sequence as DCoH (Hauer et al., 1993; Citron et al., 1992), the dimerization cofactor of hepatic nuclear transcription factor 1α (Mendel et al., 1991). This observation, together with the high rate of nonenzymatic dehydration of 4a-hydroxytetrahydropterins, raises the question of whether enzyme-catalyzed dehydration is of physiological significance.

The only source of the 4a-hydroxytetrahydropterin substrate of the dehydratase has been as the product of the reactions of aromatic amino acid hydroxylases. This has hampered investigations of the catalytic reaction of the dehydratase. Recently, we have developed a method for the chemical synthesis of 4a-hydroxytetrahydropterins (Bailey et al., 1993; S. W. Bailey, I. Rebrin, S. R. Boerth, and J. E. Ayling, manuscript submitted). The availability of chemically synthesized substrates has greatly facilitated kinetic characterization of the dehydratase and allowed an assessment of the physiological relevance of this catalytic activity, the results of which are presented here.

EXPERIMENTAL PROCEDURES

Reagents. NADH, phenylalanine, *m*-fluorotyrosine, and bovine erythrocyte superoxide dismutase (~5000 units per mg of protein) were purchased from Sigma, bovine liver catalase (50 500 units per mg of protein) from Worthington, Phenyl-Sepharose, Sephacryl, and chromatofocusing reagents from Pharmacia, hydroxylapatite from Bio-Rad, Cibacron Blue Agarose from Amicon, and 6(*R*)-tetrahydrobiopterin and 6(*R,S*)-methyltetrahydropterin from Schircks Laboratories (Jona, Switzerland). All buffer solutions used for enzyme activity assays were made to give the desired pH at each temperature and were filtered through 0.45 μm filters.

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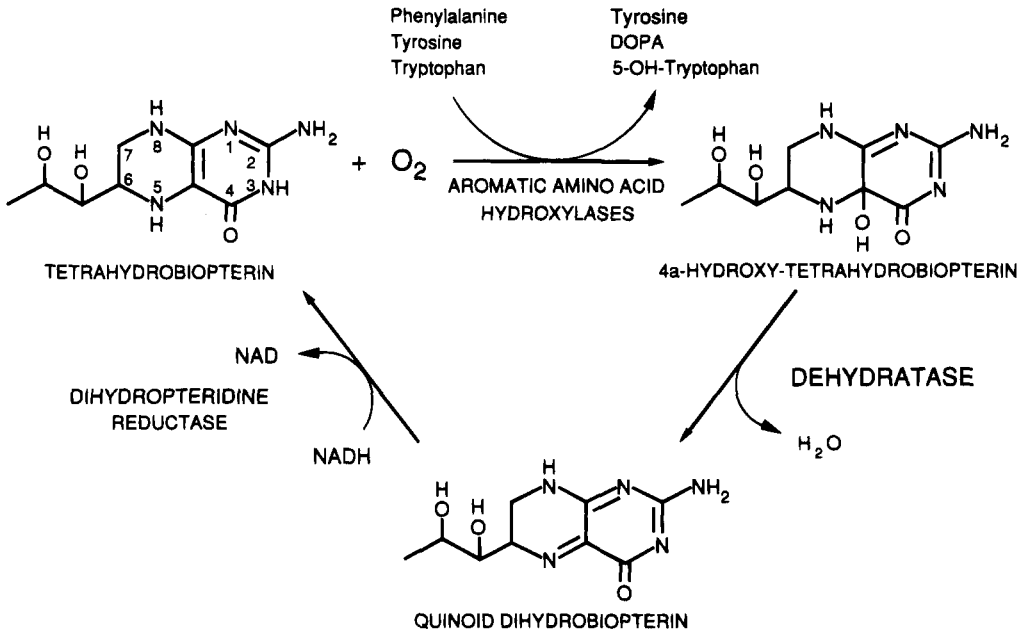
Scheme 1: Catalytic Cycle of Tetrahydrobiopterin



Dehydratase was analyzed for metal and phosphorus content by ICP; analyses were performed by the Chemical Analysis Laboratory of the University of Georgia.

Phenylalanine hydroxylase was purified from rat liver as previously published (Shiman et al., 1979). The specific activity of the enzyme, which appears >95% pure on SDS–PAGE, is 10 units per mg of protein. One unit of hydroxylase is defined as that amount which produces 1 μmol of tyrosine per minute in 0.1 M Tris-HCl, pH 7.4, at 27 °C, with 1 mM phenylalanine and 0.2 mM 6-(*R,S*)-methyltetrahydropterin. Dihydropteridine reductase was purified from bovine liver as previously (Bailey & Ayling, 1983), followed by cibacron blue affinity chromatography (Nakanishi et al., 1982). The enzyme, which appears ~95% pure on SDS–PAGE, has a specific activity of 120 units per mg of protein. A unit of reductase is defined as the amount which reduces 1 μmol of quinoid 6-methyldihydropterin per minute with 10 μM quinoid 6-methyldihydropterin and 100 μM NADH in 1 mL 0.1 M Tris-HCl, pH 7.4, at 27 °C.

4a-Hydroxytetrahydropterin Dehydratase Purification. Dehydratase was purified from rat liver by a published procedure (Huang et al., 1973) but omitting the phosphocellulose chromatography step. Human, bovine, and also rat liver dehydratase were purified by column chromatography on Phenyl-Sepharose and hydroxylapatite according to the method of Parniak (1990), followed by column chromatofocusing and gel filtration (Rebrin et al., 1992). Rat liver dehydratase was used for all experiments except where noted. Before analysis for metals and phosphorus, dehydratase was dialyzed against six changes each of 1000 volumes of 10 mM Tris-HCl, pH 7.4.

Dehydratase Activity. Enzyme activity was measured using substrate synthesized by a procedure developed in this laboratory (Bailey et al., 1993). Details of the chemical synthesis of substrates will appear elsewhere (S. W. Bailey, I. Rebrin, S. R. Boerth, and J. E. Ayling, manuscript submitted). Since the substrate is unstable, the last step in the synthesis [cyclization of 6N-(2′-alkyl-2′-aminoethyl)-quinoid divicine] is done on the same day that it is used, and a working solution of the substrate in methanol is kept in an ethanol/dry-ice bath. Rates were determined from the analysis of progress curves run to completion. Reaction mixtures containing, in a total volume of 1 mL, 0.1 μmol of NADH and an excess of dihydropteridine reductase (usually 0.35 units), in 25 mM Tris-HCl (pH 8.4 or 7.4 as specified), were temperature equilibrated in a Precision Cells type 51 water-jacketed cuvet. Initially, the absorbance at 340 nm of this mixture was monitored to ensure a stable baseline. After the desired temperature was reached, as determined with a Yellow Springs Instruments Model 729 temperature probe inserted into the mixture, 5–10 μL of 4a-hydroxytetrahydropterin was added. Upon addition of substrate there is an almost instantaneous decrease in 340 nm absorbance due to reduction by dihydropteridine reductase of the quinoid dihydropterin already present in the 4a-hydroxytetrahydropterin (S. W. Bailey, I. Rebrin, S. R. Boerth, and J. E. Ayling, manuscript submitted). This is followed by a first-order rate due to further nonenzymatic dehydration. Dehydratase was added last to the reaction mixture in an amount that completed dehydration of the substrate in 30–60 s. Data were collected with a Nelson 3000 data acquisition system (P. E. Nelson, Cupertino, CA) at a rate of 2–5 points per second from the time of addition of dehydratase until the reaction was complete. A close temporal coupling of NADH consumption to 4a-hydroxytetrahydropterin dehydration was assured by increasing the activity of DHPR to a point where no further changes in final kinetic parameters could be detected even in the fastest reactions. Typically, the amount of dihydropteridine reductase used was 5-fold the minimum amount. Dehydratase turnover is based on a subunit molecular mass of 12 000 Da and the assumption that there is one catalytic site per subunit.

Data Analysis. Progress curves were analyzed using the nonlinear fitting capabilities of MINSQ v. 4.0 or Scientist v. 2.02 (Micromath, Salt Lake City, UT) employing two different methods of calculation. The first approach, to which all data were subjected, required an endpoint for dehydration to be established. Upon completion of 4a-hydroxytetrahydropterin dehydration, as determined by HPLC analysis (S. W. Bailey, I. Rebrin, S. R. Boerth, and J. E.

Ayling, manuscript submitted), a slow nearly linear decrease in absorbance at 340 nm was noted with all substrates. With 4a-hydroxy-6-alkyltetrahydropterins, this final rate was found to be due mostly to autooxidation of the tetrahydropterin product as reflected ultimately by NADH consumption. A faster final rate was observed with 4a-hydroxytetrahydrobiopterin as substrate, mostly due to the decomposition of a byproduct formed nonenzymatically from the initial 4a-hydroxytetrahydrobiopterin (S. W. Bailey, I. Rebrin, S. R. Boerth, and J. E. Ayling, manuscript submitted). The endpoint of dehydration was established by fitting a straight line to about 30 s of data in the region clearly related only to the follow-up reaction. This line was then subtracted from all the data to produce a curve that ends with a zero rate of absorbance change at zero substrate. The concentration of 4a-hydroxytetrahydropterin during the dehydration reaction is determined from the absorbance above this endpoint using the net extinction coefficient, which at 340 nm is 5700 M^{-1} cm^{-1}. The baseline corrected curves were then fitted to the integrated form of the Michaelis–Menten equation containing an additional term for the first order rate of nonenzymatic dehydration. This employs the implicit variables function of MINSQ or Scientist as follows:

$$t = (Y - 1/k_{nonenz}) \ln[(S + Z)/(S_o + Z)] - Y \ln(S/S_o) \quad (1)$$

where: $Y = K_m/(V_m + k_{nonenz}K_m)$; $Z = V_m/k_{nonenz} + K_m$; t = time, the independent variable; S = substrate concentration at time, t, the dependent variable; S_o = substrate concentration at start of data acquisition, a parameter; K_m = Michaelis constant, a parameter; V_m = maximum velocity, a parameter; k_{nonenz} = first-order rate constant for nonenzymatic dehydration, a value determined independently (S. W. Bailey, I. Rebrin, S. R. Boerth, and J. E. Ayling, manuscript submitted) and entered into the equation.

The second approach which was applied only to 4a-hydroxy-6-alkyltetrahydropterin substrates used the differential equation fitting routine of Scientist v. 2.02. In this case data were not baseline corrected for the final residual rate (which was typically less than 1% of V_{max}) or absorbance but only converted into concentration units from the initial absorbance values. These progress curves were then fitted to

$$dS/dt = -V_m(S - S_f)/(K_m + S - S_f) - k_{nonenz}(S - S_f)$$

$$S_o = S_f + S_\Delta \quad (2)$$

where S_Δ and S_f are parameters for the change in substrate concentration over the course of the reaction and the final apparent substrate concentration (actually the NADH concentration), respectively, and the other terms are as defined in eq 1. The initial condition required for solving this differential equation has $S = S_o$ at $t = 0$. (In Scientist version 2.02 initial parameter estimates must also be entered into the body of the equation model.)

RESULTS

Kinetic Parameters as a Function of Dehydratase Concentration. A linear relationship of V_{max} with dehydratase concentration was found over the entire range examined as illustrated in Figure 1 for 4a-hydroxy-6(*S*)-propyltetrahydropterin. The K_m remained constant over this range of dehydratase except at the very lowest concentration where calculation of K_m is less certain due to the low rate, which is approaching that of the nonenzymatic reaction. A linear response to dehydratase concentration was also obtained with the 6-methyl analog.

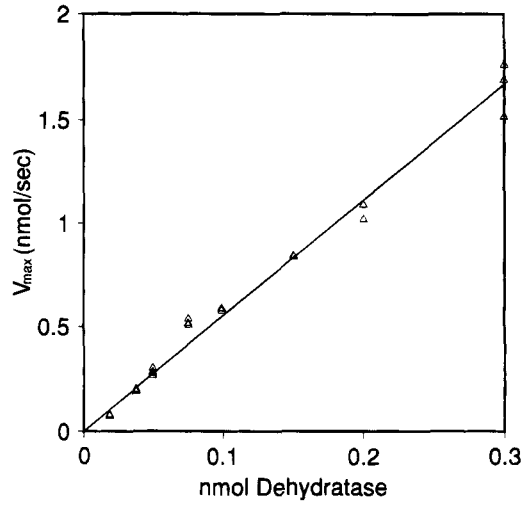


FIGURE 1: V_{max} as a function of dehydratase concentration. The substrate was 4a-hydroxy-6(*S*)-propyltetrahydropterin. Reactions were run at pH 8.4 and 10 °C. V_{max} is nmol of 4a-hydroxy-6(*S*)-propyltetrahydropterin dehydrated per second, measured as NADH consumption.

Chiral Specificity of Dehydratase. It has previously been shown that both 6-isomers of 4a-hydroxy-6(*R,S*)-methyltetrahydropterin produced by phenylalanine hydroxylase are consumed by the dehydratase (Lazarus et al., 1983), and that the 4a-hydroxy-6-methyltetrahydropterin produced by phenylalanine hydroxylase has its 4a-hydroxy group in the 4a(*S*) configuration (Dix et al., 1985). To ascertain whether the dehydratase has an absolute requirement for this configuration, chemically synthesized 4a(*R,S*)-hydroxy-6(*R,S*)-methyltetrahydropterin was tested as substrate. The enzyme rapidly catalyzed the reaction to completion, demonstrating that both 4a-isomers were substrates for the dehydratase. In order to examine the substrate properties of the individual 6-methyl isomers, 4a-hydroxytetrahydropterins derived from 6N-(2′(*R*)-aminopropyl)- and 6N-(2′(*S*)-aminopropyl)quinoid divicine were synthesized. Although the configuration of the 6-methyl group in the respective products is fixed by the precursor used, that at 4a will depend on the extent of chiral induction during cyclization (S. W. Bailey, unpublished results). 4a(*S*)-Hydroxy-6(*S*)-methyltetrahydropterin was also generated enzymatically by phenylalanine hydroxylase for comparison.

The substrate properties of the isomers were compared in dehydratase reactions at pH 7.4 and 8.4 at 10 °C. The chemically synthesized 6(*S*)-isomer and the enzymatically produced 6(*S*)-isomer gave identical results (Table 1). However, although the 6(*R*)-isomer is a good substrate, with a V_{max} similar to that of the 6(*S*) isomer, its K_m is more than twice as high as that of 6(*S*)-methyltetrahydropterin. When the substrate is a 1:1 mixture of 6(*R*) and 6(*S*)-methyl 4a-hydroxytetrahydropterin, calculation of K_m and V_{max} from the progress curve by eq 1 or eq 2 (which do not take into account competition between alternate substrates) gives erroneously high values for K_m.

Table 1: Effect of Configuration of the 6-Substituent and Method of Synthesis on the Properties of 4a-Hydroxy-6(*S*)-methyltetrahydropterin as Substrate for the Dehydratase[a]

6-methyl configuration	method of synthesis	pH 7.4		pH 8.4	
		K_m (μM)	V_{max} (s^{-1})	K_m (μM)	V_{max} (s^{-1})
6(S)	enzymatic			2.5	3.3
6(S)	chemical	2.5	9.8	2.5	3.3
6(R)	chemical	6	9.8	6	3.3

[a] Dehydratase reactions were run at 10 °C. V_{max} is expressed as turnover number, i.e., nmol of substrate dehydrated per nmol of enzyme subunit per second. The enzymatically synthesized 4a-hydroxy-6(*S*)-methyltetrahydropterin was generated as follows: Phenylalanine hydroxylase (55 μg) was incubated for 20 min with 10 mM phenylalanine in 25 mM Tris-HCl, pH 8.4, at 25 °C and then mixed with 100 μL of Phenyl-Sepharose (50% v/v suspension) in a total volume of 150 μL and incubated for a further 10 min at 4 °C. The Phenyl-Sepharose containing the bound phenylalanine hydroxylase was washed twice at 4 °C with the same buffer and then added to a solution of 30 μM 6(*S*)-methyltetrahydropterin and 1 mM phenylalanine in 25 mM Tris-HCl, pH 8.4, at 4 °C. After mixing for 2 min the Phenyl-Sepharose was quickly removed by filtration through a 3 mm diameter, 0.45 μm teflon filter (Gelman Acro LC3S), and the filtrate was used immediately as the source of 4a-hydroxytetrahydropterin.

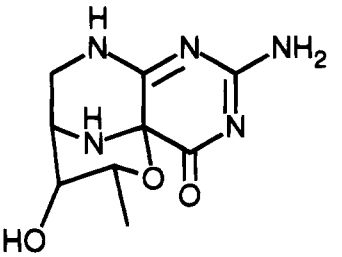
Table 2: Kinetic Parameters of 4a-Hydroxytetrahydropterins as Substrates for 4a-Hydroxytetrahydropterin Dehydratase[a]

substrate 6-substituent[b]	pH	K_m (μM)	V_{max} (s^{-1})[c]
6(*S*)-methyl-	7.4	2.5	9.8
	8.4	2.5	3.3
6(*S*)-propyl-	7.4	1.5	11.8
	8.4	1.5	5.4
6(*R*)-dihydroxypropyl-[d]	7.4	$\leq$3.0	$\geq$8.3
	8.4	$\leq$3.0	$\geq$3.3

[a] Reactions were at 10 °C. All substrates were synthesized chemically. [b] The *S* configuration of 6-methyl- and 6-propyl- is the same as the *R* configuration of 6-dihydroxypropyltetrahydropterin (tetrahydrobiopterin). [c] V_{max} is expressed as turnover number, i.e., nmol of substrate dehydrated per nmol of enzyme subunit per second. [d] The kinetic parameters for 4a-hydroxy-6(*R*)-tetrahydrobiopterin are approximate due to interference from the nonenzymatically formed O2′,4a-cyclic-6(*R*)-tetrahydrobiopterin.

Effect of 6-Substituent on the Substrate Properties of 4a-Hydroxytetrahydropterins. The substrate properties of chemically synthesized 4a-hydroxy-6(*S*)-methyltetrahydropterin, 4a-hydroxy-6(*S*)-propyltetrahydropterin, and 4a-hydroxy-(6*R*)-tetrahydrobiopterin were compared using progress curve analysis at 10 °C in order to minimize the background rate due to nonenzymatic dehydration. All were found to be good substrates with K_m values of 1.5−3 μM which were independent of pH (7.4 or 8.4). V_{max} values at pH 7.4 (8−12 s^{-1}) were 2−3 times faster than at pH 8.4 (Table 2). K_m and V_{max} values for 4a-hydroxytetrahydrobiopterin cannot be determined with high accuracy since a byproduct, believed to be O2′,4a-cyclic-6(*R*)-tetrahydrobiopterin, is formed nonenzymatically during the reaction (see below), leading to a relatively high terminal rate which interferes with the analysis. This situation was encountered whether 4a-hydroxytetrahydrobiopterin was synthesized chemically or enzymatically.

Substrate Properties of O2′,4a-Cyclic-6(R)-tetrahydrobiopterin. When the nonenzymatic dehydration of 4a-hydroxy-6(*R*)-tetrahydrobiopterin (in the presence of NADH and dihydropteridine reductase) is monitored by HPLC, another pteridine product can be detected in addition to tetrahydrobiopterin. This compound, which at pH 8.4 and 10 °C is more stable than 4a-hydroxy-6(*R*)-tetrahydrobiopterin, has been tentatively identified as O2′,4a-cyclic-6(*R*)-tetrahydrobiopterin (S. W. Bailey, I. Rebrin, S. R. Boerth, and J. E. Ayling, manuscript submitted). To generate enough of this material to determine whether it is a substrate for the dehydratase, a solution of 80 μM 4a-hydroxy-6(*R*)-tetrahydrobiopterin in 1.0 mL of 25 mM Tris-HCl, pH 8.4, containing the standard amounts of NADH and dihydropteridine reductase was allowed to nonenzymatically dehydrate for 25 min at 10 °C. At this time an amount of dehydratase was added which instantly catalyzed dehydration of any remaining 4a-hydroxy-6(*R*)-tetrahydrobiopterin. The O2′,4a-cyclic-6(*R*)-tetrahydrobiopterin which remained was completely consumed in 40 s with the utilization of about 6 μM NADH. At this concentration of O2′,4a-cyclic-6(*R*)-tetrahydrobiopterin, the initial rate of the dehydratase catalyzed reaction was 1−2% of that of a control reaction containing 6 μM 4a-hydroxy-6(*R*)-tetrahydrobiopterin.



O2′,4a-cyclic-6(*R*)-tetrahydrobiopterin

Kinetic Parameters as a Function of pH and Temperature. Kinetic parameters for the enzyme-catalyzed reaction were determined as a function of temperature from 3 to 37 °C at both pH 8.4 and 7.4 with 4a-hydroxy-6(*S*)-methyltetrahydropterin and 4a-hydroxy-6(*S*)-propyltetrahydropterin as substrate. There is very little effect of pH on the Michaelis constants. However, from 10 to 37 °C, K_m increases by about 2-fold for all substrates.

The maximum velocities (k) as a function of temperature are plotted in Figure 2. As can be seen from these graphs, increasing temperature has a greater effect on the reaction of the 6(*S*)-propyl analog than on 4a-hydroxy-6(*S*)-methyltetrahydropterin. However, both substrates display linear Arrhenius plots at both pH's (Figure 2, inset). Therefore, there appears to be no conformational change of the enzyme over the temperature range of 4 to 37 °C, either at pH 7.4 or 8.4, or if there is one, it does not affect the rate of reaction. Data acquired with 4a-hydroxy-(6*R*)-tetrahydrobiopterin are not shown, since interference by non-enzymatic formation of the O2′,4a-cyclic-6(*R*)-tetrahydrobiopterin precludes accurate analysis.

The enthalpy (ΔH^*) and entropy (ΔS^*) of activation of the dehydratase catalyzed reactions, calculated from the data in Figure 2, are summarized in Table 3. The larger 6(*S*)-propyl substituted analog was found to have a higher ΔH^*, especially at pH 7.4, than 4a-hydroxy-6(*S*)-methyltetrahydropterin. On the other hand, the former has a more favorable ΔS^* that compensatates (at 310 K) for the enthalpy disadvantage to make enzymatic dehydration of 4a-hydroxy-6(*S*)-propyltetrahydropterin nearly 2-fold faster. These differences are due to their differential interaction with the dehydratase, since the thermodynamic parameters for their nonenzymatic dehydration are indistinguishable. With both

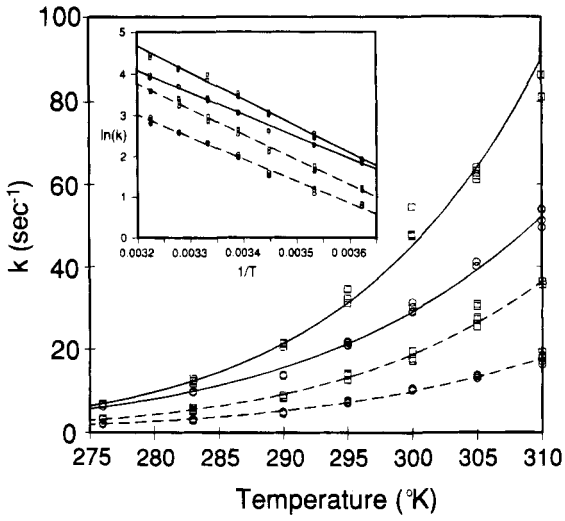


FIGURE 2: Dehydratase turnover vs temperature with 4a-hydroxy-6(*S*)-methyltetrahydropterin (○), or 4a-hydroxy-6(*S*)-propyl-tetrahydropterin (□) as substrate at pH 7.4 (—), or 8.4 (- - -). The lines are nonlinear least-squares fit of the data (weighted in inverse proportion to k) to $k = (RT/N\hbar)e^{\Delta S^*/R}e^{-\Delta H^*/RT}$, where k = the rate constant (nmol of 4a-hydroxytetrahydropterin dehydrated per nmol of dehydratase monomer per second at saturating substrate concentration), R = the gas constant, N = Avogadro's number, $\hbar$ = Planck's constant, and T = temperature (K). The inset shows an Arrhenius plot of the same data (fitted with no weighting).

Table 3: Thermodynamic Parameters for the Dehydration of 4a-Hydroxytetrahydropterins

4a-hydroxy-tetrahydropterin	pH	ΔH^* (kcal mol^{-1})	ΔS^* (cal mol^{-1} K^{-1})	ΔG^* [a] (kcal mol^{-1})
		Dehydratase catalyzed[b]		
6(*S*)-methyl-	7.4	10.14	-18.05	15.74
	8.4	10.25	-19.81	16.39
6(*S*)-propyl-	7.4	12.21	-10.29	15.40
	8.4	11.64	-13.91	15.95
		Nonenzymatic[c]		
6(*S*)-methyl-	7.4	12.07	-26.16	20.18
	8.4	15.05	-17.47	20.47
6(*S*)-propyl-	7.4	12.06	-26.02	20.13
	8.4	14.85	-18.08	20.45

[a] ΔG^* is calculated for 310 K. [b] Calculated from the turnover data in Figure 2. [c] Data from S. W. Bailey, I. Rebrin, S. R. Boerth, and J. E. Ayling (manuscript submitted).

substrates almost all of the increase in rate of the enzymatic reaction at pH 7.4, in comparison to pH 8.4, is due to an increase in ΔS^*. This is in contrast to nonenzymatic dehydration where the differences in ΔH^* and ΔS^* are due to a change in mechanism from one that is primarily solvent catalyzed (at pH 8.4) to a reaction dominated by catalysis by proton (pH 7.4).

The ratios of enzymatic turnover to nonenzymatic first-order rate constant are plotted in Figure 3 as a function of temperature for each substrate at each pH. At pH 8.4, with 6-propyl and with 6-methyl, the catalytic efficiency of the enzyme decreases with increase in temperature. With 6-methyl at pH 7.4, there is a similar relationship, although less pronounced. However, with 6-propyl at pH 7.4 the ratio is constant over the temperature range of 4−37 °C, with a catalytic efficiency of 2100.

Metal and Phosphorus Content. EDTA in concentrations up to 0.1 M had no effect on enzyme activity. Analysis for metal content showed that rat and bovine dehydratase

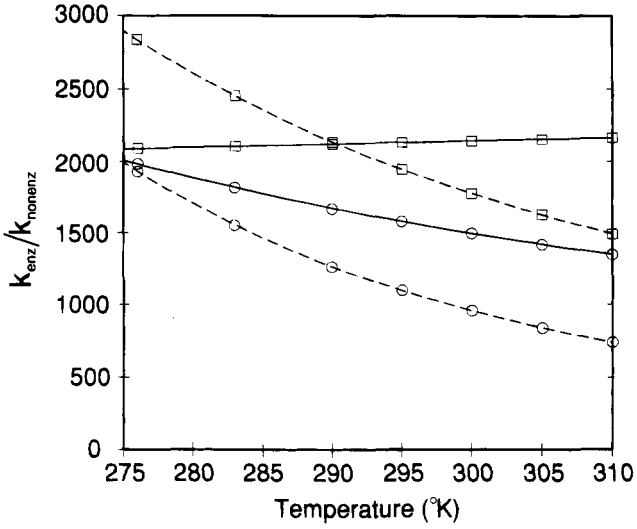


FIGURE 3: Ratio of enzymatic turnover/nonenzymatic rate constants as a function of temperature at pH 7.4 (—), or 8.4 (- - -), for 4a-hydroxy-6(*S*)-methyltetrahydropterin (○), or 4a-hydroxy-6(*S*)-propyltetrahydropterin (□).

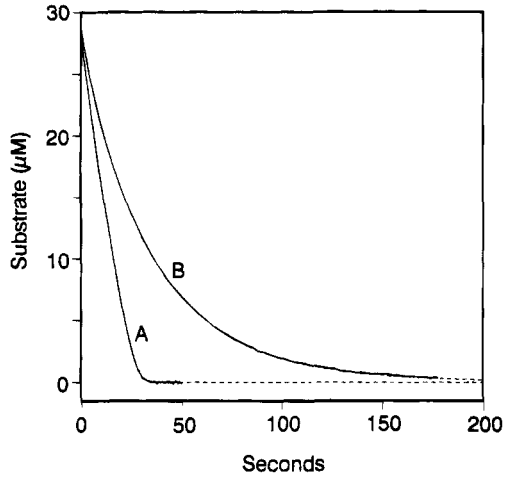


FIGURE 4: Progress curves of dehydratase reactions with 4a-hydroxy-6(*S*)-methyltetrahydropterin as substrate. The reactions contained 0.14 nmol of dehydratase/mL and were run at pH 7.4 and 10 °C. Dehydratase was added at zero time. (A) Standard reaction mixture containing NADH and dihydropteridine reductase. (B) NADH and dihydropteridine reductase were omitted resulting in a decrease in rate due to product inhibition.

contained 0.03−0.05 atoms of Mg and less than 0.03 atoms of Zn, Cu, Fe, Al, Mn, Mo, Co, Ni, Cr, or Ca per subunit. Human dehydratase contained 0.02 atoms of Mg and less than 0.01 atoms of the other metals per subunit. No phosphorus was detected at a sensitivity which could have measured less than 0.01 atoms per subunit. There was no effect of the dialysis which preceded these analyses on the kinetic parameters of the enzyme.

Product Inhibition. The progress of a dehydratase reaction with 4a-hydroxy-6(*S*)-methyltetrahydropterin as substrate in the presence of NADH and an excess of dihydropteridine reductase is shown in Figure 4 (curve A). In the absence of NADH and dihydropteridine reductase, dehydratase reactions decelerated more rapidly than could be accounted for by consumption of substrate (Figure 4, curve B). This was found to be due to inhibition by the product, quinoid dihydropterin. Quantitation of the inhibition by quinoid dihydropterins necessitates omitting NADH and dihydropteridine reductase from the reaction mixture. Therefore,

Table 4: Inhibition of Dehydratase by Quinoid Dihydropterins, Tetrahydropterins, and 7,8-Dihydrobiopterin[a]

inhibitor	type of inhibition	K_I (μM)
quinoid 6(*S*)-methyldihydropterin	comp.	1.5 ± 0.1
quinoid 6(*S*)-propyldihydropterin	comp.	0.7 ± 0.1
7,8-dihydrobiopterin	none	no inhibition <0.2 mM
6(*R*)-tetrahydrobiopterin	none	no inhibition <0.2 mM
6(*S*)-methyltetrahydropterin	none	no inhibition <1.0 mM

[a] Reactions were at pH 7.4 and 10 °C. Inhibition by quinoid 6(*S*)-propyldihydropterin was quantitated with 4a-hydroxy-6(*S*)-propyltetrahydropterin as substrate. For all other compounds tested as inhibitors, 4a-hydroxy-6(*S*)-methyltetrahydropterin was substrate. Product inhibition by quinoid 6(*R*)-dihydrobiopterin was not quantitated due to the instability of 4a-hydroxy-6(*R*)-tetrahydrobiopterin and quinoid 6(*R*)-dihydrobiopterin.

reactions were monitored by directly observing the formation of quinoid dihydropterins. Although the maximum differences in extinction between the substrate and product are at 246 and 325 nm, neither of these wavelengths were suitable for monitoring the reaction, since quinoid dihydropterins tautomerize to 7,8-dihydropterins. Therefore, the isosbestic point of these two dihydropterins at 340 nm was used. Reactions were initiated by simultaneous addition of varying amounts of 4a-hydroxytetrahydropterin and the corresponding quinoid dihydropterin to the reaction cuvet containing temperature equilibrated dehydratase in 25 mM Tris-HCl, pH 7.4, at 10 °C. The amount of quinoid dihydropterin present at zero time was calculated from the initial decrease in 340 nm absorbance after addition of the same amount of 4a-hydroxytetrahydropterin and quinoid dihydropterin to a parallel reaction which contained NADH and dihydropteridine reductase instead of dehydratase. The type of inhibition was determined by plotting the reciprocal of the initial rate vs I_o/S_o according to the method of Freudenthal (1970). While this showed that quinoid dihydropterins were competitive inhibitors, accurate values for K_I were difficult to obtain due to the increasing concentration of quinoid dihydropterin as the reaction proceeded (Table 4).

Inhibition constants were obtained from progress curves of reactions taken to completion in the absence of NADH and dihydropteridine reductase. An amount of dehydratase was used to complete the reaction in about 3 min, or less. During this time at 10 °C in 25 mM Tris-HCl at pH 7.4, tautomerization of quinoid 6-methyl- or 6-propyldihydropterin to the corresponding 7,8-dihydropterin was less than 1%. Kinetic parameters were then calculated from the integrated rate equation for competitive product inhibition, in the presence of simultaneous nonenzymatic dehydration of substrate:

$$P = S_o - S + M_o$$

where S is evaluated from the equation

$$t = (K_m U/X - 1/k_{nonenz}) \ln[(X + WS)/(X + WS_o)] - (K_m U/X) \ln(S/S_o) \quad (3)$$

where $U = (K_I + S_o + I_o)$; $W = k_{nonenz}(K_I - K_m)$; $X = K_I V_m + K_m k_{nonenz} U$; P, the dependent variable = the concentration of product at time, t, plus M_o; t, S, S_o, K_m, V_m, and k_{nonenz} have the same meaning as in eq 1; K_m is determined independently (see above) and is entered as a fixed value; and I_o, K_I, and M_o are additional parameters, where I_o = initial inhibitor concentration, K_I = inhibition constant, and M_o = initial absorbance, divided by the net extinction coefficient. The inhibition constants determined by this procedure are summarized in Table 4. From these results it is evident that the product of the dehydratase reaction binds tightly to the enzyme, the K_I values being even lower than the K_m's for the respective substrate.

The effects of tetrahydropterins and 7,8-dihydropterins on dehydratase activity were determined in a reaction mixture containing 0.1 μmol of NADH, 0.35 units of dihydropteridine reductase, and dehydratase in 25 mM Tris-HCl, pH 7.4, at 10 °C. These compounds were added immediately before substrate, and the reaction was monitored to completion. In contrast to quinoid dihydropterins, progress curve analysis showed no inhibition by 7,8-dihydrobiopterin or tetrahydrobiopterin in concentrations up to 0.2 mM, the highest concentrations that could be used without interference with the assay, or by 6(*S*)-methyltetrahydropterin up to 1 mM (Table 4).

Effect of Phenylalanine Hydroxylase on Dehydratase Activity. Previously, data have been acquired with dehydratase substrate, 4a-hydroxytetrahydropterin, generated in the reaction mixture by phenylalanine hydroxylase. The following experiments were performed to ascertain whether phenylalanine hydroxylase itself has any effect on the activity of the dehydratase. Dehydratase, 0.15 nmol, was incubated for 2 h on ice with varying amounts of phenylalanine hydroxylase (0, 0.01, 0.1, 0.45, and 0.9 nmol) in 10 μL of 25 mM Tris-HCl, pH 7.4. All of this mixture was then used to catalyze the dehydration of chemically synthesized 4a-hydroxy-6(*S*)-methyltetrahydropterin at pH 7.4 and 10 °C. There was no effect of phenylalanine hydroxylase on either K_m or V_{max} even when present in a 6-fold molar excess over dehydratase.

Effect of Ionic Strength and Solvent on Dehydratase Activity. KCl, in increasing concentrations, was added to a dehydratase reaction at pH 8.4 and 10 °C with 4a-hydroxy-6(*S*)-methyltetrahydropterin as substrate. Over a range of 0.02−2 M there was no effect of KCl on the K_m or the rate of the enzymatic reaction.

Addition of ethylene glycol inhibited the reaction. As ethylene glycol was increased up to 25% (w/v), V_{max} decreased linearly. Extrapolation to 100% ethylene glycol gave a V_{max} of zero. Dehydratase was not inactivated by ethylene glycol even after 20 h incubation at 4 °C in 50% ethylene glycol in 25 mM Tris-HCl, pH 8.4. The nonenzymatic rate of dehydration was unaffected by concentrations of ethylene glycol up to 50%.

Does 4a-Hydroxytetrahydropterin Cause Product Inhibition of Aromatic Amino Acid Hydroxylases? The possibility that 4a-hydroxytetrahydropterin may cause product inhibition of the hydroxylases which generate it was investigated with phenylalanine hydroxylase utilizing chemically synthesized 4a-hydroxytetrahydropterins. To ensure that the phenylalanine hydroxylase preparation was free of dehydratase activity, the rate of decay of 4a-hydroxy-6-methyltetrahydropterin was monitored in the absence and presence of phenylalanine hydroxylase. Concentrations of phenylalanine hydroxylase used in the following reactions (up to 0.16 units/mL), had no significant effect on the rate of dehydration of 4a-hydroxytetrahydropterins.

The activity of phenylalanine hydroxylase was monitored from the tyrosine produced (Bailey & Ayling, 1980) as a function of time in the presence and absence of varying concentrations of 4a-hydroxy-6(*R*,*S*)-methyltetrahydropterin. In order to minimize decay of the 4a-hydroxy-6-methyltetrahydropterin, reactions were run in 25 mM Tris-HCl, pH 8.4, at 17 °C. Reaction mixtures containing phenylalanine hydroxylase (0.016 units/mL), 2 mM phenylalanine, 0.5 mM NADH, dihydropteridine reductase (3.4 units/mL), catalase (2500 units/mL), and superoxide dismutase (250 units/mL), in 25 mM Tris-HCl, pH 8.4, were incubated for 5 min at 27 °C to activate the enzyme followed by 3 min at 17 °C to temperature equilibrate the solution. Reactions were initiated by the addition of the cofactor, 6(*R*,*S*)-methyltetrahydropterin (0.3 mM). After 2 min, 4a-hydroxy-6(*R*,*S*)-methyltetrahydropterin was added. A cofactor concentration of approximately $4K_m$ was used in order to minimize any increase in rate due to recycling of quinoid 6(*R*,*S*)-methyldihydropterin arising from the spontaneous dehydration of 4a-hydroxy-6(*R*,*S*)-methyltetrahydropterin. The reaction progress was monitored before and after addition of 4a-hydroxy-6(*R*,*S*)-methyltetrahydropterin. Control reactions were identical except that an equivalent amount of 4a-hydroxy-6(*R*,*S*)-methyltetrahydropterin which had been allowed to decay at 17 °C for 18 min (approximately 7 half-lives) was added instead at 2 min of reaction. No inhibition by 4a-hydroxy-6(*R*,*S*)-methyltetrahydropterin was detected with concentrations of up to 0.8 mM. To ascertain whether this lack of inhibition could be due to a mechanism in which the cofactor product is released before tyrosine, the effect of 4a-hydroxytetrahydropterin was also determined in the presence of a high concentration (0.4 mM) of a product analog, *m*-fluorotyrosine. *m*-Fluorotyrosine was chosen for this purpose since it can be easily separated from *p*-tyrosine in the HPLC analysis for tyrosine. Also, *m*-fluorophenylalanine is a good substrate for phenylalanine hydroxylase (J. E. Ayling, unpublished results). Again no inhibition by 4a-hydroxy-6(*R*,*S*)-methyltetrahydropterin was observed.

Since the natural cofactor, 6(*R*)-tetrahydrobiopterin, possesses specific regulatory properties with phenylalanine hydroxylase, similar experiments were performed with chemically synthesized 4a-hydroxy-6(*R*)-tetrahydrobiopterin. Further, since the binding of 4a-hydroxytetrahydropterins to phenylalanine hydroxylase may be affected by their charge on N5, the effect of 4a-hydroxy-6(*R*)-tetrahydrobiopterin on the reaction of phenylalanine hydroxylase was determined at pH 7.4 as well as at pH 8.4. In order to obtain rates which are linear with time for extended periods, the phenylalanine hydroxylase was not phenylalanine activated. Reaction mixtures contained 20 μM 6(*R*)-tetrahydrobiopterin ($4K_m$), phenylalanine hydroxylase (0.16 units/mL), catalase, and superoxide dismutase in 25 mM Tris-HCl, pH 7.4, and were temperature equilibrated to 17 °C before initiation with phenylalanine (1 mM). A rate was established by monitoring tyrosine formation for 2 min before addition of 4a-hydroxy-6(*R*)-tetrahydrobiopterin. Still no inhibition of hydroxylase activity was seen at any concentration of 4a-hydroxy-6(*R*)-tetrahydrobiopterin ranging from 0.05 to 0.4 mM whether or not *m*-fluorotyrosine was included in the reaction mixture. Thus, even with the natural cofactor and at physiological pH, phenylalanine hydroxylase is not inhibited by its cofactor product, 4a-hydroxy-6(*R*)-tetrahydrobiopterin.

DISCUSSION

Since the initial discovery of a protein capable of stimulating the overall hydroxylation of phenylalanine under certain *in vitro* conditions (Kaufman, 1970), the physiological significance of this finding has remained uncertain. This protein was later shown to have 4a-hydroxytetrahydropterin dehydratase activity (Huang & Kaufman, 1973; Lazarus et al., 1983). However, the need for such an activity is not usually manifest in typical *in vitro* hydroxylase assays, except those performed near pH 8.4, where 4a-hydroxytetrahydropterins are maximally stable. The role of the "dehydratase" has been further complicated by the finding that its sequence of 103 amino acids is completely identical to that of DCoH, a dimerization cofactor for the transcription regulator HNF-1α (Mendel et al., 1991). There is very little documentation of other proteins having two such disparate functions as this. However, a cytoplasmic iron regulated RNA binding protein has been reported which has the identical sequence to cytosolic aconitase (Kaptain et al., 1991; Kennedy et al., 1992). This protein can alternate between these two activities dependent on how many of its iron−sulfur clusters are intact (Haile et al., 1992; Philpott et al., 1993). So far, there is no analogous explanation for the two functions of the dehydratase/DCoH protein. The results of the current investigation, and those concerning the nonenzymatic reactions (S. W. Bailey, I. Rebrin, S. R. Boerth, and J. E. Ayling, manuscript submitted), have clarified the importance of the 4a-hydroxytetrahydropterin dehydratase activity in phenylalanine metabolism.

Although it is possible to examine dehydratase activity by directly monitoring the initial rate of absorbance change accompanying transformation of a 4a-hydroxytetrahydropterin to the corresponding quinoid dihydropterin, the combination of a high degree of product inhibition (see below) and the relatively low K_m for substrate give rates that are quite nonlinear with time. This problem, which hinders measurement of accurate enzyme kinetic parameters, was alleviated by coupling the dehydratase reaction with a large excess of dihydropteridine reductase to give a tetrahydropterin (which is not a dehydratase inhibitor) as final product. Since many data points can be collected in the region of low substrate concentration, progress curve analysis using an equation that takes nonenzymatic dehydration into account further improves the measurement of micromolar K_m values. The accuracy of K_m's and rates determined by this approach is ultimately limited by spectrophotometric noise in relation to the net extinction coefficient (NADH $\rightarrow$ NAD^+ plus 4a-hydroxytetrahydropterin $\rightarrow$ tetrahydropterin) and by any uncertainty of the parallel nonenzymatic rate which is determined in a separate reaction. The importance of the latter factor diminishes with increased dehydratase activity, i.e., short times to completion of reaction. The major factor limiting the detection of low levels of enzyme activity is the relative rate of the spontaneous reaction. Although there are no significant differences between the nonenzymatic rates of dehydration of 6(*S*)-propyl and 6(*S*)-methyl 4a-hydroxytetrahydropterins, at pH 8.4 the former has a fairly large differential $\Delta H^*_{nonenz} - \Delta H^*_{enz}$ while also having a relatively low ΔS^*_{enz}. The advantage (k_{enz}/k_{nonenz}) of the propyl analog is especially apparent in reactions performed between 0 and 10 °C.

The method of 4a-hydroxytetrahydropterin synthesis is of importance in obtaining 4a-hydroxytetrahydropterins suitable for use as substrates for the dehydratase. The use of excess oxidant in the synthesis of dehydratase substrates must be avoided, otherwise enzyme assays give erroneous results, probably due to formation of halogen/pteridine adducts. When such substrates are used, the fit of progress curves to the data are poor and reactions do not end sharply. The use of stoichiometric bromine and catalytic $K_3Fe(CN)_6$ solves this problem (S. W. Bailey, I. Rebrin, S. R. Boerth, and J. E. Ayling, *manuscript submitted*).

At 37 °C and pH 7.4, V_{max}/K_m for dehydration of 6(*S*)-methyl- and 6(*S*)-propyl 4a-hydroxytetrahydropterins (about 1×10^7 M^{-1} s^{-1} and 3×10^7 M^{-1} s^{-1}, respectively) are within a factor of 10 of the highest values observed for other enzymes. Since V_{max}/K_m is not completely at the diffusion limit, the possibility remains that the dissociation constant for substrate might be close to the measured K_m. A K_I of 43 μM (assuming competitive inhibition) at 21 °C for the relatively stable substrate analog 4a-hydroxy-5-deaza-6(*R,S*)-methyltetrahydropterin has been reported (Lazarus et al., 1983). The current results show that the V_{max} of dehydration is unaffected and K_m only doubled when using 4a-hydroxy-6(*R*)-methyltetrahydropterin in comparison to the naturally configured 6(*S*)-isomer (Table 1). Therefore, the greater than 10-fold decrease of affinity of the deaza compound in comparison to the pterin analog appears to be primarily due to the lack of the 5-nitrogen (and/or its associated proton and lone electron pair).

The dehydratase shows little sensitivity to side-chain structure of its pterin substrate, in contrast to the aromatic amino acid hydroxylases. For example, while K_m for the tetrahydropterin cofactor with phenylalanine hydroxylase or protein-kinase-A-phosphorylated tyrosine hydroxylase decreases by a factor of about 30 or 130, respectively, on extending the 6-substituent of tetrahydropterin from 6(*S*)-methyl- to 6(*S*)-propyl (Bailey et al., 1991), less than a 2-fold difference in K_m was seen with the analogous 4a-hydroxy-6-alkyltetrahydropterins as dehydratase substrates. Further, although nonenzymatic intramolecular cyclization of the side chain of 4a-hydroxytetrahydrobiopterin limits the accuracy of the determination of its K_m, the estimated value (which is if anything too high) suggests that the 1′- and 2′-hydroxyl groups do not decrease affinity relative to an unsubstituted propyl group to the extent that occurs with the hydroxylases. This relative lack of side-chain specificity also appears in the affinities for 6(*S*)-methyl- and 6(*S*)-propylquinoid dihydropterins which are competitive product inhibitors. Studies of nonenzymatic carbinolamine dehydration kinetics indicates considerable advancement toward product in the transition state in terms of C−O bond cleavage (Sayer et al., 1973; Sayer & Jencks, 1977), although not necessarily accompanied by concomitant C−N hybridization to sp^2 (Archilla, et al., 1971). The observations that the 6(*S*)-alkylquinoid dihydropterin product analogs have low K_I values (about half of the K_m for their respective 4a-hydroxy substrate analogs) suggest that the dehydratase may recognize product and transition state in a similar manner. The degree of product inhibition revealed by the 6(*S*)-alkylquinoid dihydropterins shows that high physiological levels of dihydropteridine reductase are useful for preventing accumulation not only of quinoid dihydrobiopterin but also of 4a-hydroxytetrahydrobiopterin. An alteration of the protein fluorescence of human dehydratase by tetrahydrobiopterin has been observed (Rebrin et al., 1993). Since the current work finds no inhibition of dehydratase activity by 6(*R*)-tetrahydrobiopterin up to 0.2 mM, the reported fluorescence changes upon association of 6(*R*)-tetrahydrobiopterin with dehydratase must be unrelated to activity. Alternatively, the fluorescence changes may have been due to contaminating quinoid dihydropterins.

Several dehydratases in the hydrolyase class, including aconitase, contain iron−sulfur clusters, which act as Lewis acids in the dehydration reaction. These enzymes have high stereospecificity for their substrates. In the present study, no metal was found associated with 4a-hydroxytetrahydropterin dehydratase in any significant quantity and there is only one cysteine per subunit. The most likely mechanisms for enzyme-catalyzed dehydration of 4a-hydroxytetrahydropterins are those which have been demonstrated for the analogous nonenzymatic reaction, which include general acid catalysis (S. W. Bailey, I. Rebrin, S. R. Boerth, and J. E. Ayling, manuscript submitted). Further, any proposed mechanism of the enzymatic reaction must take into account the observation that both synthetic 6(*S*)-methyl and 6(*R*)-methyl 4a-hydroxytetrahydropterin were completely consumed by the dehydratase. In neither case was the progress curve biphasic, indicating that both 4a-isomers are good substrates. Preliminary molecular modeling studies show that the hydroxyl group on the tetrahedral 4a-carbon is considerably out of the plane of the conjugated atoms in the pyrimidine moiety of the substrate. A 4a(*S*)- and 4a(*R*)-hydroxyl group would therefore occupy markedly different positions in the catalytic site. If the mechanism of the dehydratase involves acid catalysis, then the amino acid residue involved must have considerable flexibility. Alternatively, proton donation might take place via an intervening water molecule which could position itself to accommodate the configuration of the 4a-hydroxyl group. The linear pattern of inhibition by ethylene glycol, in which the decrease in rate is directly proportional to the percent ethylene glycol, is consistent with an essential role for water in enzyme catalysis.

Catalysis of the nonenzymatic reaction by buffer base has also been observed, but in a manner suggesting an action only on the neutral species (S. W. Bailey, I. Rebrin, S. R. Boerth, and J. E. Ayling, manuscript submitted). Base catalysis of dehydration by the enzyme would resolve the lack of 4a-stereospecificity, since the protein base could interact with one of the protons left on N8, N3, or the 2-amino group (the tautomeric structure of 4a-hydroxytetrahydropterins is not yet known) which are likely located in similar positions in both 4a-isomers. Additionally, a mechanism involving both acid and base catalysis by the dehydratase is also conceivable in which the activated intermediate is a zwitterion.

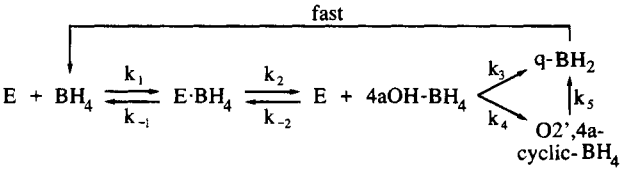
In *in vitro* assays of phenylalanine hydroxylase at neutral pH and especially in the presence of high concentrations of monoanionic phosphate, the rate of nonenzymatic dehydration at 27 °C, or above, may be sufficient to maintain almost all cofactor in its reduced state (in the presence of excess dihydropteridine reductase). Estimates of the activity of phenylalanine hydroxylase *in vivo* indicate that, under a load of phenylalanine, the rate of hydroxylation in the liver can exceed 5 μM s^{-1} (Bailey et al., 1993; Lipton et al., 1967; Antonas et al., 1974; Youdim et al., 1975; Milstien & Kaufman, 1975). At this level of activity, the cytoplasmic environment does not support a rate of nonenzymatic

dehydration capable of maintaining the pool of reduced 6(*R*)-tetrahydrobiopterin, which is present at a concentration of about 6 nmol/g of rat liver. Based on the results presented here, the concentration of dehydratase in rat liver is calculated to be 6 μM (subunit). Therefore, the rate in the liver would be about 324 μM s^{-1}, assuming that at 37 °C the kinetic parameters of 4a-hydroxytetrahydrobiopterin are similar to those of the 6(*S*)-methyl analog, as is the case at 10 °C. Even with the high rate of hydroxylation under a phenylalanine load, this level of dehydratase activity is adequate to keep the concentration of 4a-hydroxytetrahydrobiopterin to less than 2% of the total liver pool of 6(*R*)-tetrahydrobiopterin. Thus, not only is the dehydratase necessary physiologically, but its activity appears sufficient to fill the need. Moreover, the very high V_{max}/K_m values for substrate imply that the protein has been nearly optimized for this reaction.

In addition to maintaining the pool of reduced cofactor, the dehydratase may also prevent production of potentially detrimental byproducts of 4a-hydroxytetrahydrobiopterin decomposition. 7-Tetrahydrobiopterin and related derivatives, which have been detected in low concentrations in normal individuals, are greatly increased in patients with a variant form of hyperphenylalaninemia (Curtius et al., 1988), hypothesized to be due to dehydratase deficiency (Curtius et al., 1990; Davis et al., 1991). This regioisomer has been shown to be an inhibitor of phenylalanine hydroxylase (Davis et al., 1992; Adler et al. 1992). Formation of 7-tetrahydrobiopterin from 6(*R*)-tetrahydrobiopterin during hydroxylase reactions lacking the dehydratase has been demonstrated. It has been suggested that this occurs by ring-opening of the accumulating 4a-hydroxytetrahydrobiopterin followed by rearrangement and recondensation (Curtius et al., 1990; Davis et al., 1991; Adler et al., 1992). The feasibility of such a mechanism is supported by the generation of some 7-isomer during the cyclization of 6N-(2′-alkyl-2′-aminoethyl)quinoid divicines, especially in entirely aqueous solvent (S. W. Bailey, I. Rebrin, S. R. Boerth, and J. E. Ayling, manuscript submitted). The need to prevent 7-isomer formation may, therefore, be a factor accounting for the high level of dehydratase activity found in liver.

Another possible role for the dehydratase is to prevent accumulation of 4a-hydroxytetrahydrobiopterin, which could itself be an inhibitor. Since it is the immediate product of the hydroxylases, as well as an analog of the putative 4a-hydroperoxide intermediate, it has considerable potential to cause product inhibition of the aromatic amino acid hydroxylases. In earlier studies with purified phenylalanine hydroxylase, it was observed that, in 40 mM potassium phosphate, pH 8.0, the apparent K_m for 6(*R,S*)-tetrahydrobiopterin increased with an increase in hydroxylase concentration (Huang & Kaufman, 1973). One interpretation of this effect, which was specific to tetrahydrobiopterin as cofactor, was that it was due to competition with tetrahydrobiopterin for the hydroxylase by the accumulating 4a-hydroxytetrahydrobiopterin product. However, in addition to the lack of detectable inhibition of phenylalanine hydroxylase by 4a-hydroxytetrahydropterins reported here, we have observed that the nonenzymatic rates of dehydration of 4a-hydroxy-6(*R*)-tetrahydrobiopterin and 4a-hydroxy-6-methyltetrahydropterin are similar to each other (Bailey et al., 1995). These results can be reconciled with the earlier report by the mechanism outlined in Scheme 2. This scheme is identical to that previously employed (Huang & Kaufman, 1973), except for inclusion of the steps representing formation of the putative tetrahydrobiopterin O2′,4a-cyclic adduct (k_4) and its breakdown (k_5) (S. W. Bailey, I. Rebrin, S. R. Boerth, and J. E. Ayling, manuscript submitted). In 40 mM potassium phosphate, pH 8.0, the O2′,4a-cyclic adduct is almost an order of magnitude more stable than 4a-hydroxytetrahydrobiopterin. Modeling the pre-steady-state and steady-state phases of the reaction including this adduct (using the differential equation capability of Scientist version 2.0) predicts an increase in apparent K_m with increase in hydroxylase concentration, without the necessity of invoking product inhibition. The cyclic adduct, which can be formed by tetrahydrobiopterin, but not by 6-methyltetrahydropterin, also explains why the apparent product inhibition was specific to tetrahydrobiopterin.

Scheme 2: Effect of O2′,4a-Cyclic-6(*R*)-tetrahydrobiopterin on the Kinetics of Phenylalanine Hydroxylase[a]



[a] E, phenylalanine hydroxylase; BH_4, tetrahydrobiopterin; 4a-OH-BH_4, 4a-hydroxytetrahydrobiopterin; q-BH_2, quinoid dihydrobiopterin.

In conclusion, the dehydratase appears to play an essential role in phenylalanine metabolism: (1) to ensure, together with dihydropteridine reductase, a continuous supply of reduced cofactor, and (2) to prevent accumulation of potentially harmful species produced by rearrangement of 4a-hydroxytetrahydrobiopterin.

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