

Hyperphenylalaninemia and 7-Pterin Excretion Associated with Mutations in 4a-Hydroxy-tetrahydrobiopterin Dehydratase/DCoH: Analysis of Enzyme Activity in Intestinal Biopsies

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Hyperphenylalaninemia, which can cause neurological disorders and mental retardation, results from a mutation in phenylalanine hydroxylase or an enzyme required for biosynthesis or regeneration of its cofactor, tetrahydrobiopterin. The hyperphenylalaninemia variant primapterinuria is characterized by the excretion of 7-biopterin (primapterin). This disorder is thought to be due to a deficiency of 4a-hydroxy-tetrahydrobiopterin dehydratase (pterin-4a-carbinolamine dehydratase), but a lack of tissue activity has not been directly demonstrated. The five mutations so far recognized in patients with primapterinuria are associated with either a single amino acid change or a premature stop codon. Only C81R has been successfully expressed in soluble form, and was found to have 40% of normal activity. Tissues which could be obtained by minimally invasive procedures were analyzed for dehydratase activity. None was detected in normal human white cells or fibroblasts. However, activity was found in intestine of rat, dog, pig, and particularly humans where it was only eight times lower than in liver. Distribution along the length and across the wall of small intestine was relatively uniform. Moreover, the dehydratases from human liver and intestinal mucosa have identical kinetic properties. A biopsy of duodenal mucosa from a patient with homozygous E96K dehydratase had activity of 55 nmol · min⁻¹ · g⁻¹ mucosa compared to 329 ± 32 nmol · min⁻¹ · g⁻¹ tissue in controls (*n* = 12). The sixfold lower tissue activity of the E96K mutant alone may not be

sufficient to account for the biochemical symptoms of primapterinuria in this patient. However, accumulation of a 4a-hydroxy-tetrahydrobiopterin degradation product (a side-chain cyclic adduct), which has been observed *in vitro* and appears to be a dehydratase inhibitor, may further exacerbate the problem. © 2000

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The most common cause of hyperphenylalaninemia is a deficiency in phenylalanine hydroxylase which results in phenylketonuria (PKU) and, if not treated, severe mental retardation. Less frequently, hyperphenylalaninemia is due to deficiency in an enzyme involved in tetrahydrobiopterin² biosynthesis or regeneration. Tetrahydrobiopterin is an essential cofactor for phenylalanine, tyrosine, and tryptophan hydroxylases (1) (Fig. 1) and the NO synthases (2). The three aromatic amino acid hydroxylases utilize tetrahydrobiopterin to activate molecular oxygen to produce tyrosine, dopa, and 5-hydroxytryptophan, respectively. Tetrahydrobiopterin is regen-

² Abbreviations used: tetrahydrobiopterin; 6(*R*)-*L*-erythro-dihydroxypropyl-5,6,7,8-tetrahydropterin; 7-biopterin, 7-*L*-erythro-dihydroxypropyl-pterin; 7-neopterin, 7- δ -*threo*-trihydroxypropyl-pterin; 6-keto-7-biopterin, 6-keto-7-*L*-erythro-dihydroxypropyl-pterin; tetrahydropterin, 2-amino-4-keto-5,6,7,8-tetrahydropteridine; Tris, tris-(hydroxymethyl)aminomethane; PCD, pterin-4a-carbinolamine dehydratase.

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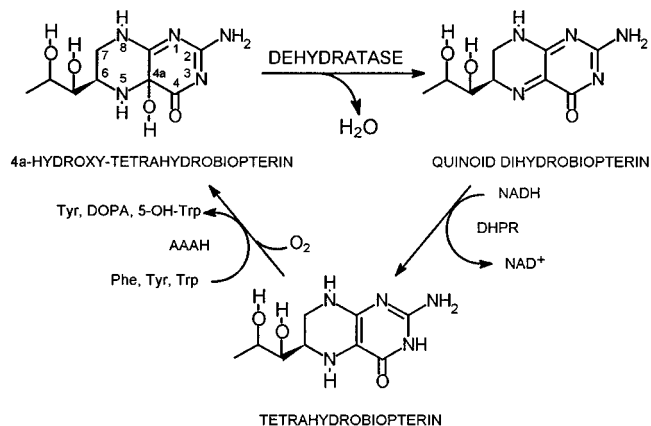


FIG. 1. Role of 4a-hydroxy-tetrahydrobiopterin dehydratase in the biosynthesis of catecholamines and serotonin, and in the catabolism of phenylalanine. AAAH, aromatic amino acid hydroxylases; DHPR, dihydropteridine reductase.

erated from the cofactor product of the reaction, 4a-hydroxy-tetrahydrobiopterin, by the successive reactions catalyzed by 4a-hydroxy-tetrahydrobiopterin dehydratase (EC 4.2.1.96) (3,4) and dihydropteridine reductase (1) (Fig. 1). In a variant form of hyperphenylalaninemia known as primapterinuria, the plasma phenylalanine usually approaches normal levels by 2 years of age, but persistent high levels of 7-biopterin as well as 7-neopterin and 6-keto-7-biopterin are excreted in the urine (5–8) (Fig. 2). It was hypothesized that this variant form could be due to a deficiency of 4a-hydroxy-tetrahydrobiopterin dehydratase. In support of this hypothesis, it was shown that 7-pterins can be produced from tetrahydrobiopterin in a reaction of phenylalanine hydroxylase in the absence of the dehydratase (9,10).

4a-Hydroxy-tetrahydrobiopterin dehydratase is a homotetramer with 103 amino acids in each subunit (11,12). Analysis of the dehydratase gene of a child with the symptoms of this disorder revealed a point mutation corresponding to C81R in one allele and a termination mutation, E86X, in the other (13). To determine the effect of these mutations on the catalytic activity of the dehydratase, C81R and E86X were cloned and expressed in *Escherichia coli* (14,15). The C81R dehydratase was found to have about 40% of the activity of the wild-type with no change in K_m (14–16). When E86X was expressed recombinantly, most of the protein was found in the insoluble fraction so that activity could not be quantitatively determined (14,17). Although some activity was detected (17), dehydratase with only the first 85 N-terminal amino acids would be expected to

have insignificant activity since it lacks R87 which forms a salt link with E80 at the edge of the catalytic site. Even if the activity of E86X is insignificant, the patient might be expected to have 20% of normal activity from the C81R allele. However, recombinant C81R was shown to be more susceptible than wild-type to proteolysis as determined by the rapid loss of activity when incubated with trypsin or cathepsin and by the rapid decrease in the dehydratase band when analyzed by SDS-PAGE. It was suggested that this decreased stability could result in decreased enzymatic activity *in vivo*, in addition to that due to the direct effect of the mutation on catalytic activity (14).

To further test the hypothesis that hyperphenylalaninemia associated with high urinary levels of 7-biopterin was due to deficiency of 4a-hydroxy-tetrahydrobiopterin dehydratase, the sequence of the dehydratase gene from several other patients was determined. Mutations were found corresponding to T78I, Q97X (17), E96K, and E26X (18). The first three of these, T78I, Q97X, and E96K, were cloned and expressed in *E. coli*. In all cases, the majority of the dehydratase was found in the insoluble fraction. Enough Q97X and E96K remained in the soluble fraction to allow determination of dehydratase activity. The mutant dehydratase, E96K, was found to have a K_m for substrate identical to that of wild-type, whereas the K_m for substrate of Q97X was only two to three times higher. However, the amount of either of these mutant dehydratases in the soluble fraction was too small to allow purification and determination of specific catalytic activity (17,18).

Since the actual level of dehydratase activity in the tissues of these patients is unknown, these data leave open the question of whether mutations in the dehydratase are (i) the sole cause of the excretion of

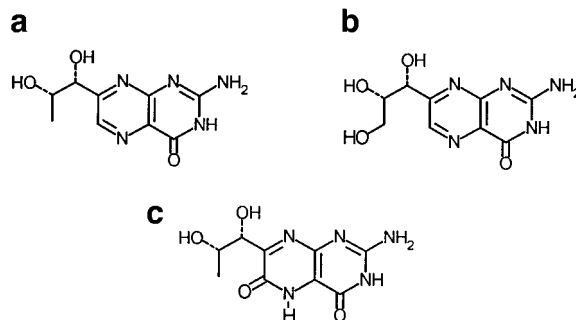


FIG. 2. Degradation products of tetrahydrobiopterin in the absence of 4a-hydroxy-tetrahydrobiopterin dehydratase: (a) 7-biopterin, (b) 7-neopterin, and (c) 6-keto-7-biopterin.

high levels of 7-pterins and transient hyperphenylalaninemia, or (ii) a contributing factor, or (iii) incidental polymorphisms. We have found that in addition to liver and kidney, there is significant activity of 4a-hydroxy-tetrahydrobiopterin dehydratase in the intestine. Since intestinal biopsies are routinely performed for diagnosis of GI disorders, this presented a means of directly comparing dehydratase activity in the tissue of a patient with that in controls.

MATERIALS AND METHODS

Materials. The dehydratase substrates, 6(*R*)-4a-hydroxy-tetrahydrobiopterin, 6(*S*)-propyl-4a-hydroxy-tetrahydropterin, and 6(*S*)-methyl-4a-hydroxy-tetrahydropterin, were stereospecifically synthesized as described elsewhere (4,19). Human liver dehydratase, which has identical amino acid sequence to the enzyme from rat liver, and dihydropteridine reductase were purified as previously reported (20). A unit of reductase is defined as the amount which reduces 1 μ mol of quinoid 6-methyl-dihydropterin per minute with 10 μ M quinoid 6-methyl-dihydropterin and 100 μ M NADH in 1 mL of 0.1 M Tris-HCl, pH 7.4, at 27°C. Buffer solutions were made to give the desired pH at the temperature utilized. Cell culture medium (Dulbecco's Modified Eagle's Medium, DMEM) was from Gibco Life Technologies (Rockville, MD) (#12100). All other reagents were from Sigma (St. Louis, MO).

Animal tissues. Anesthetized animals were perfused with 0.15 M NaCl and tissues were removed and washed with 0.15 M NaCl. A section of small intestine was taken from a pig and the mucosal layer removed by lightly scraping with a metal spatula. From the dog, 2-inch segments of duodenum, jejunum, and ileum were collected. The mucosal layer was removed from a portion of each section, and the remainder was stored intact. All tissues were immediately frozen in liquid nitrogen and stored at -80°C until extracted and analyzed.

Human tissues. Freshly prepared and washed buffy coat was used as the source of human white cells. The washed white cells were resuspended in an equal volume of buffer and frozen in liquid nitrogen. Human skin fibroblasts from a 6-month-old female were grown in DMEM supplemented with 10% fetal calf serum. Cells from 22 plates (approx. 10^8 cells) were washed with phosphate-buffered saline, collected by centrifugation, and the cell pellet was resuspended in 0.5 mL distilled water, and frozen in

liquid nitrogen. Human liver and duodenum were obtained from organ donors who were traffic accident victims. Human ileum was obtained from a patient undergoing ileostomy. Biopsies of duodenal mucosa were obtained by endoscopy as part of routine diagnostic procedures for GI disorders. A portion of the biopsy, 4-12 mg, was frozen in liquid nitrogen immediately upon removal.

Patient. Hyperphenylalaninemia was detected in a patient (BIODEF #306) (21,22) in a newborn screening test. His urine was found to contain high levels of 7-biopterin (0.8 mmol/mol creatinine compared to 0.02-0.058 in controls). Sequencing of the gene revealed a mutation corresponding to E96K, which has previously been reported in another unrelated patient (BIODEF #216) (18). As mentioned above, when cloned and expressed most of the mutant dehydratase was in the insoluble fraction. Some activity was detected in the 100,000-g supernatant but at a level which was too low to allow quantitative evaluation, except for the determination of K_m , which was found to be the same as wild-type (18). The tissue analyzed in the current work was a biopsy of duodenal mucosa obtained from patient BIODEF #306 at 2 years of age.

Extraction of tissues. Tissues were homogenized in a Potter-Elvehjem homogenizer on ice in 2 mL activity assay buffer per gram tissue. All samples of complete cross sections of intestine, and other fibrous tissues, were first pulverized under liquid nitrogen to facilitate homogenization. Human fibroblasts and human white cells were homogenized by hand in a microground glass homogenizer. Homogenates were centrifuged at 100,000g for 60 min at 4°C and the supernatants used as the source of dehydratase. Biopsies of human intestine mucosa were weighed in Kontes homogenization microfuge tubes while still frozen, 125 μ L of activity assay buffer was added, and the tissues were homogenized by hand on ice. Homogenates were spun at 4°C in a microfuge at 15,000g for 30 min. From the supernatant 10 μ L was taken for determination of protein (23), and 100 μ L was immediately analyzed for dehydratase activity.

Measurement of dehydratase activity. Activity was measured using chemically synthesized substrate (19) by a procedure which we have published (4,20). Reaction mixtures were assembled in a water-jacketed 1 cm light-path microcuvet through which cold water was circulated to maintain the cuvet contents at 10°C. Temperature was deter-

mined with a Yellow Springs Instrument (Yellow Springs, OH) Model 729 temperature probe inserted into the reaction mixture. Standard reactions were run in 25 mM Tris-HCl, pH 8.4 (or pH 7.4 where noted), at 10°C, and contained NADH (0.1 mM) and an excess of dihydropteridine reductase (0.5 units). After addition of a 100,000g supernatant of homogenate as the source of dehydratase, the reaction was monitored at 340 nm for any nonspecific oxidation of NADH. When a stable baseline was reached (typically 2–5 min), the dehydratase reaction was initiated by addition of 7.5 μL of 2 mM 6(*S*)-propyl-4a-hydroxy-tetrahydropterin substrate to a total reaction volume of 1 mL. The accuracy of the initial substrate concentration is not critical since reactions are run to completion and kinetic parameters are determined by analysis of the complete progress curve. Data were acquired by computer at 2 points/s and the reaction velocity and K_m calculated as previously published (20,24). The nonenzymatic rate of dehydration under these conditions is 0.0022 s^{-1} and is taken into account in the calculations.

Measurements of activity in biopsies of duodenal mucosa from children were made in 10 mM Tris-HCl, pH 8.2, in order to obtain the highest ratio of enzymatic to nonenzymatic rate, and thereby increase sensitivity. Under these conditions the enzymatic rate is 14% faster and the nonenzymatic rate 15% slower than in 25 mM Tris-HCl, pH 8.4. The sensitivity was further increased by masking the cuvet so that reactions could be run in a total volume of 0.28 mL, in this case initiated with 2.2 μL of 2 mM substrate. Each biopsy was analyzed at least three times. During the reaction, the product quinoid dihydropterin is immediately reduced by NADH and dihydropteridine reductase to tetrahydropterin, which does not interfere with the dehydratase (20). Thus, when sample is limited, repeat assays can be performed in the same reaction mixture by running the reaction to completion and then repeating the addition of NADH and substrate to initiate a new reaction.

RESULTS

Evaluation of human white blood cells and human skin fibroblasts for dehydratase activity. Human white cells were analyzed for dehydratase activity with extract of white cells from 5 mL up to 50 mL of blood added to the reaction. No dehydratase activity was detected even at the highest amount added. Similar analyses were performed with extracts from

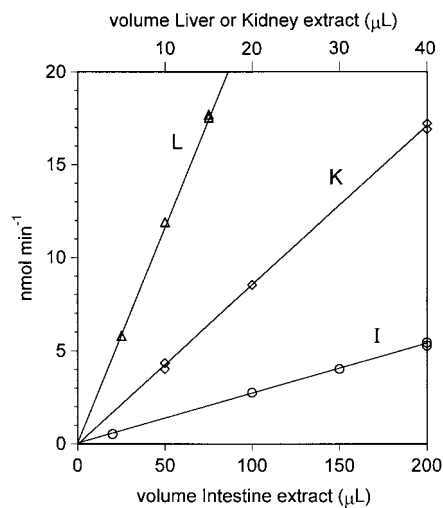


FIG. 3. 4a-Hydroxy-tetrahydrobiopterin dehydratase activity as a function of the amount of 100,000-g supernatants of homogenates of rat tissues (1 g tissue: 2 mL assay buffer). Reaction volume, 1 mL. L, liver; K, kidney; I, small intestine.

human skin fibroblasts over the range of extract from 10^7 cells (0.2 mg protein) to 4×10^7 cells (0.8 mg protein) in a 1-mL reaction. Again, no dehydratase activity could be detected.

Distribution of dehydratase activity in rat tissues. Initial experiments to determine tissue distribution of dehydratase were conducted in rat. Rat dehydratase has the identical amino acid sequence to the human enzyme. In order to ascertain the validity of measurements of dehydratase activity in crude 100,000-g supernatants, the relationship between amount of tissue extract in the reaction and dehydratase activity was determined. In all cases a linear relationship between V_{max} and milligram wet weight of tissue was observed over the range measured (Fig. 3), indicating the lack of any significant inhibition by endogenous compounds. From these rates the concentration of dehydratase in different tissues was calculated based on the turnover values (micromoles product formed per micromole enzyme per minute) which we have previously established for homogeneously pure rat liver dehydratase under similar activity assay conditions (20). The levels of dehydratase in different rat tissues are summarized in Table 1. The relative activities in liver, kidney, pancreas, and lung are consistent with those previously reported (25). In addition, we also found dehydratase in rat intestine. The activity in a sampling of total rat small intestine is higher than in all other tissues except for liver and kidney. Immunohisto-

TABLE 1
Distribution of Dehydratase in Rat

Tissue	Concentration ^a of dehydratase (μM)
Liver	11
Kidney	4
Small intestine (all sections)	0.25
Pancreas	0.2
Lung	0.15
Adrenal (whole)	0.1
Brain stem	0.15

^a Concentration of the 12,000-Da subunit.

chemical techniques have detected dehydratase containing cells throughout the various layers of the rat intestine (26).

Dehydratase activity in the intestine of other species and in different sections of the intestine. To ascertain whether dehydratase activity in the intestine is specific for rats or is a more general phenomenon, samples of intestine were obtained from dog and pig. Activity was found in the small intestine of both species. The distribution of dehydratase activity along the intestine and across the intestinal wall was determined in dog tissues. Enzymatic activity was found to be similar in jejunum, duodenum, and ileum. Interestingly, in all three sections the activity in the mucosa is similar to that in the underlying tissue (Table 2). The K_m for substrate, 6(*S*)-propyl-4a-hydroxy-tetrahydropterin, determined with dog and pig mucosa was found to be the same as for rat and human enzyme (see below). Assuming that the turnover (micromoles product formed per micromole enzyme per minute) is also the same as for rat and

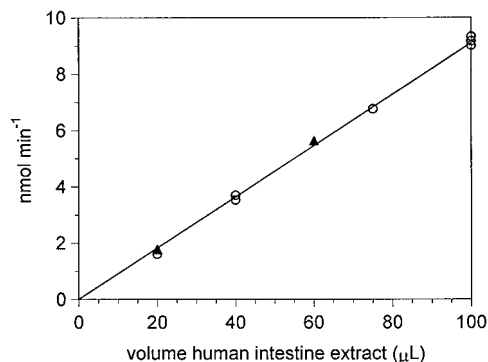


FIG. 4. 4a-Hydroxy-tetrahydrobiopterin dehydratase activity as a function of the amount of 100,000-g supernatants of homogenates of human ileum (▲) or human duodenal mucosa (○) (1 g tissue: 2 mL assay buffer). Reaction volume, 1 mL.

human (20), the dehydratase concentration in the small intestine of dog and pig is around $0.4 \mu\text{M}$.

Characterization of human intestine dehydratase. Dehydratase activity in human intestine was measured with different volumes of intestine extract. A linear relationship between activity and volume of extract was observed (Fig. 4). The concentration of dehydratase in human intestine calculated from the data in Fig. 4 is $0.88 \mu\text{M}$. The same result was obtained whether the extract was from ileum removed at surgery or was duodenal mucosa removed from an organ donor. Similar experiments with human liver gave a concentration of dehydratase in human liver of $7.2 \mu\text{M}$ ($n = 4$).

Dehydratase in human intestine extracts was characterized kinetically and compared with purified human liver enzyme. The relative activity of intestine dehydratase with three different substrates, 6(*S*)-methyl-4a-hydroxy-tetrahydropterin, 6(*S*)-propyl-4a-hydroxy-tetrahydropterin, and 6(*R*)-4a-hydroxy-tetrahydrobiopterin, was the same as that observed for the liver enzyme, as was the ratio of V_{max} at pH 7.4 to V_{max} at pH 8.4. The K_m for substrate with the intestine enzyme was also the same as with the liver enzyme (Table 3). Thus, it appears that the dehydratase in the intestine is the same as the liver enzyme and therefore can be used as a source of dehydratase for evaluating the effect of mutations on the catalytic activity of the enzyme.

Dehydratase activity in intestine biopsies from children. Duodenal mucosa biopsies were obtained by endoscopy from 12 children as part of routine diagnostic procedures for GI disorders. These samples were used to determine the extent of variability

TABLE 2
Distribution of Dehydratase in Dog Intestine

Intestine section	Concentration ^a of dehydratase (μM)
Duodenum ^b	0.42
Duodenum mucosa	0.42
Jejunum ^b	0.4
Jejunum mucosa	0.39
Ileum ^b	0.39
Ileum mucosa	0.41

^a Concentration of the 12,000-Da subunit.

^b Entire cross section. Values are the average of two to four measurements. The concentration of dehydratase in dog liver was found to be $32 \mu\text{M}$.

TABLE 3
Comparison of Kinetic Properties of Human Intestine Mucosa Dehydratase with Human Liver Dehydratase

Parameter	Substrate	pH	Intestine	Liver
V_{\max} ratio	6(S)-propyl-4a-OH-PH ₄ /6(S)-methyl-4a-OH-PH ₄	8.4	1.6	1.6
V_{\max} ratio	6(R)-4a-OH-BH ₄ /6(S)-methyl-4a-OH-PH ₄	8.4	1	1
V_{\max} ratio	6(S)-methyl-4a-OH-PH ₄	7.4/8.4	3	3
V_{\max} ratio	6(S)-propyl-4a-OH-PH ₄	7.4/8.4	2.2	2.2
K_m	6(S)-propyl-4a-OH-PH ₄	7.4 and 8.4	1.0 μ M	1.0 μ M
K_m	6(S)-methyl-4a-OH-PH ₄	7.4 and 8.4	1.5 μ M	1.5 μ M

Note. 4a-OH-PH₄, 4a-hydroxy-tetrahydropterin; 4a-OH-BH₄, 4a-hydroxy-tetrahydrobiopterin.

among children. Each biopsy was analyzed with at least three progress curves, with an average intra-assay standard deviation of $\pm 4\%$. As can be seen from the results (Table 4, Fig. 5), there is very little variation among children from 1 to 14 years of age. The average of 12 children is 329 ± 32 nmol min⁻¹ (gram tissues)⁻¹ (Fig. 5A, Table 4), and 4.84 ± 0.35 nmol min⁻¹ (mg protein)⁻¹ (Fig. 5B, Table 4). The average of the activity found in adult small intestine (Fig. 4) is the same as the average of these children.

Effect of a mutation in 4a-hydroxy-tetrahydrobiopterin dehydratase on its activity in a biopsy sample. The dehydratase in human duodenal mucosa appears to have the same properties as that in liver (Table 3, see above). Therefore, the activity was measured in a biopsy of duodenal mucosa obtained from a patient with a homozygous dehydratase mutation (E96K). Since the activity found in the biopsies from the 12 control children is very uniform regardless of age, sex, or the disorder for which they

TABLE 4
Dehydratase Activity in Biopsies of Duodenal Mucosa

Age (months)	Sex	Weight of biopsy (mg)	Specific activity (nmol/min/mg protein)	Activity (nmol/min/g tissue)	Concentration ^a (μ M) dehydratase in duodenal mucosa	
Controls						
12	F	4.1	5.35	374	1.02	
17	M	7.43	4.59	323	0.87	
26	M	5.5	4.81	298	0.81	
30	M	5.6	5.29	325	0.88	
60	M	5.05	5.08	342	0.92	
73	M	5.16	4.88	341	0.92	
123	M	8.52	4.47	298	0.81	
128	F	9.95	4.15	295	0.80	
143	M	8.53	5.14	390	1.05	
147	F	12.15	4.66	298	0.81	
148	F	7.39	4.91	358	0.88	
172	M	9.3	4.74	310	0.84	
			Average	4.84 ± 0.35	329 ± 32	0.88 ± 0.08
E96K mutation						
24	M	3.52	0.85	55	0.15	

^a Concentrations are of the 12-kDa subunit and are calculated from the average of three activity measurements. The turnover (nmol product/min/nmol dehydratase, 12 kDa) is 370 with 6(S)-propyl-4a-hydroxy-tetrahydropterin as substrate in 10 mM Tris-HCl, pH 8.2, at 10°C. Only one child, the 26-month-old, was found to have a disorder of the intestine (celiac disease). The 123-month and 172-month-old were diagnosed as gastritis, and the 148-month-old, esophagitis. No other disorders were detected.

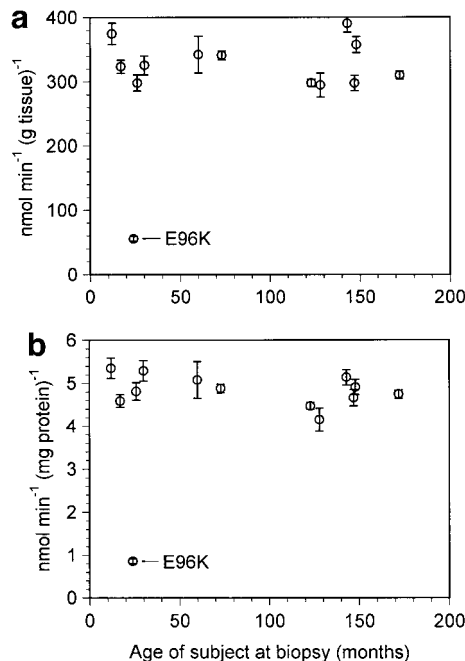


FIG. 5. 4a-Hydroxy-tetrahydrobiopterin dehydratase activity in duodenal biopsies of 12 control children and a child homozygous for an E96K mutation in his dehydratase. Activities, nanomoles product formed per minute are (A) per gram tissue, and (B) per milligram protein. The activity in each sample was measured at least three times. Activities are plotted as the average \pm SD.

were being diagnosed (Table 4, Fig. 5), the male patient (who was 2 years of age at the time the biopsy was taken) can be compared to their average value. As can be seen from Table 4 and Fig. 5, the dehydratase activity in duodenal mucosa of the patient with the homozygous E96K mutation is about 17% of that found in controls based on milligram wet weight of tissue and 17.6% based on activity per milligram protein. The K_m of the dehydratase from the E96K patient was found to be the same as for controls, consistent with the value determined with the poorly soluble recombinant E96K dehydratase expressed in *E. coli* (18).

DISCUSSION

The small intestine of different species was found to contain significant amounts of dehydratase activity with relatively uniform distribution of activity along the length and across the wall of the intestine. Human small intestine mucosa dehydratase was found to have identical kinetic properties to the liver enzyme. Furthermore, dehydratase activity in duodenal mucosa biopsies did not vary greatly among

controls. Therefore, intestinal mucosa appears to be a suitable tissue for assessing the dehydratase status of an individual.

Using duodenal mucosa biopsies, a patient homozygous for the dehydratase variant, E96K, was found to possess tissue activity levels of about one-sixth of normal. Glutamate 96 is not within the catalytic site, but it forms an ionic bond with K35 on an adjacent β -sheet in the same subunit. Disruption of this bond could affect activity either directly or by a change of conformation. Consistent with the latter possibility is the inability to express E96K in soluble form in *E. coli* (18), in contrast to wild-type dehydratase which remains soluble even at a level of expression equivalent to 30% of the total soluble protein of *E. coli* (24).

The activity of another dehydratase mutation, C81R, was measured *in vitro* and found to contain 40% of normal activity (14–16). Considering this level of activity, plus the assumption that an E86X mutation would produce inactive dehydratase, it was calculated that a patient with a compound heterozygous mutation, C81R/E86X, would have 20% of normal activity. Since this level of activity was deemed insufficient to cause the biochemical symptoms of this patient, it was proposed that tissue levels might be further decreased by twofold or more due to a higher than normal susceptibility of C81R to proteolysis (14). However, the results presented here, which for the first time measure the actual tissue activity of a mutant dehydratase, demonstrate that a patient with primapterinuria has 17% of the normal level.

The majority of tetrahydrobiopterin utilization is in the liver as cofactor for phenylalanine hydroxylase in the catabolism of phenylalanine. We have shown that dehydratase activity in rat liver is sufficient to maintain 99% of tetrahydrobiopterin in the reduced form, even during a phenylalanine load (4). A similar conclusion can be made for normal human liver, since although dehydratase in human liver is somewhat lower than in rat, phenylalanine hydroxylase activity is also lower (27). 4a-Hydroxy-tetrahydrobiopterin that is not either enzymatically or spontaneously dehydrated (19) can degrade by at least two routes: formation of 7-tetrahydrobiopterin (presumably via 7-quinoid dihydrobiopterin) (9,10,19), and formation of a side-chain 4a-cyclic adduct of tetrahydrobiopterin (Fig. 6) (19,20). Except in the extreme case where the steady-state concentration of 4a-hydroxy-tetrahydrobiopterin approaches K_m for the dehydratase ($<3 \mu\text{M}$, [20]), a

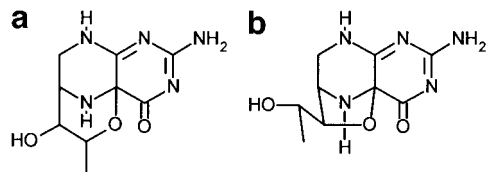


FIG. 6. 4a-Cyclic adduct produced from 4a-hydroxy-tetrahydrobiopterin by displacement of the 4a-hydroxyl group, (a) by the 2'-hydroxyl of the 6 substituent (19,20), or (b) by the 1'-hydroxyl of the 6-substituent. Preliminary data were consistent with structure (a) (19,20), but we have since shown that 6-hydroxymethyl-4a-hydroxy-tetrahydrobiopterin can form an analogous adduct. Therefore, structure (b) cannot be excluded.

simple analysis suggests that the rate of 7-biopterin production would depend linearly on the level of dehydratase activity (assuming that the rate constant of the decomposition pathways are unchanged in the patient). Thus, one would expect a 6-fold increase in 7-biopterin excretion in the patient with the E96K mutation. Instead, a 13- to 40-fold increase is observed: 0.8 mmol/mol creatinine in the patient compared to 0.02 to 0.058 mmol/mol creatinine in controls (17,28).

The difference between the expected and observed excretion of 7-biopterin into the urine might be accounted for by the action of the side-chain 4a-cyclic adduct of tetrahydrobiopterin. The dehydratase binds the cyclic adduct and converts this to quinoid dihydrobiopterin, but at a rate more than 50-fold slower than that of 4a-hydroxy-tetrahydrobiopterin (19,20). Preliminary data suggest that the cyclic adduct has an affinity similar to that of 4a-hydroxy-tetrahydrobiopterin. Dehydratase progress curves with 4a-hydroxy-tetrahydrobiopterin as substrate show that an inhibitor is produced *in vitro* as the reaction proceeds. A decrease in dehydratase activity due to a mutation would cause accumulation of the cyclic adduct, which is about 10 times more stable than 4a-hydroxy-tetrahydrobiopterin (19,20). This will further inhibit the dehydratase, reinforcing not only its own production, but also that of 7-pterins. Thus, the effect of the mutation would be compounded. The influence of the cyclic adduct on the analysis of dehydratase activity in biopsy samples would be minimal, since if any remained after homogenization and centrifugation, it would be diluted at least 100-fold in the activity assay. The dehydratase mutation, and the inhibition of the remaining dehydratase activity in the liver by the 4a-cyclic adduct of tetrahydrobiopterin, may be suf-

ficient to account for the biochemical symptoms of these patients.

Patients with primapterinuria typically excrete about half of the biopterin as 6-biopterin and half as 7-biopterin. Since these patients have hyperphenylalaninemia at birth, it appears that degradation decreases liver tetrahydrobiopterin to an extent not sufficient to maintain the necessary rate of phenylalanine hydroxylation. However, most patients eventually establish normal blood phenylalanine levels, even though continuing to produce 7-pterins (17,18). This observation is not unexpected since, for example, if the liver pool of tetrahydrobiopterin is decreased during phenylalanine metabolism to 90% of normal due to lower recycling by the dehydratase, this would not greatly affect phenylalanine hydroxylase activity. Conversely, having 10% of the pool as 4a-hydroxy-tetrahydrobiopterin would result in a significant increase of 7-biopterin formation. Urinary 7-pterin levels, therefore, are a more sensitive means of detecting dehydratase deficiency than is the plasma phenylalanine concentration. Heterozygotes for dehydratase, which presumably have at least half of normal activity, excrete normal or only slightly elevated amounts of 7-pterins (8,28). Therefore, in contrast to individuals with 17% of full dehydratase activity, it appears that 50% is adequate for maintaining tetrahydrobiopterin mostly in the reduced state.

4a-Hydroxy-tetrahydrobiopterin dehydratase has a second function as DCoH, dimerization cofactor of the transcription factor HNF1 α (11,12,29). As a dehydratase, the protein is a soluble cytoplasmic enzyme of four identical subunits of 12,000 Da. As DCoH, two of the 12,000-Da subunits combine with two subunits of HNF1 α to form an active transcription complex. A large number of genes are regulated by HNF1 α , including phenylalanine hydroxylase (30). Other disorders which may be related to deficiency in HNF1 α function, such as renal Fanconi syndrome (31), have not been reported for patients with the E96K or other mutations in this protein. Therefore, either dehydratase catalytic activity is not required for DCoH function, or 17% of normal activity is adequate. Also, if there is a conformational change in the protein caused by the mutation, this has not been observed to have any serious effect on DCoH activity.

The variation in dehydratase activity in tissues of different species (Table 5) may be a reflection of the different diets of each species. Since dogs are primarily carnivores, the high activity of dehydratase

TABLE 5
Concentrations of Dehydratase (μM) in Liver and Small Intestine of Different Species

Species	Liver	Intestine	Liver/intestine
Rat	11	0.25	45
Dog	32	0.4	80
Human	7.2	0.88	8

observed in the liver would be required to maintain the tetrahydrobiopterin cofactor for phenylalanine hydroxylase in the metabolism of phenylalanine. On the other hand, the intestinal dehydratase observed in this study may play a role in regenerating tetrahydrobiopterin for tryptophan hydroxylase, the rate limiting enzyme in the biosynthesis of serotonin. Tryptophan hydroxylase has been localized immunohistochemically in human, rat (32,33), and mouse (34) intestinal wall and mucosa, although activity is 40-fold lower in mouse intestine than in mouse brain (35). On the other hand, we have shown that dehydratase activity in rat intestine is similar to that in rat brain stem (Table 1). Serotonin is required in the GI tract for normal digestive function (36). The relatively high dehydratase in human intestine may explain why patients with as low as 17% of normal activity do not experience GI dysfunction. Since intestinal mucosa is a rapidly regenerating tissue, the high level of dehydratase, which appears to be in large excess over that needed to support tryptophan hydroxylase, could be related to the DCoH transcriptional function of the protein.

Biopsy of intestinal mucosa is a relatively noninvasive procedure. For those enzymes which are not expressed in a more readily accessible cell type, analysis of intestinal mucosa samples may provide a means of assessing the effect of mutations on the level of activity *in vivo*.

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