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7-SUBSTITUTED PTERINS: FORMATION DURING PHENYLALANINE HYDROXYLATION IN THE ABSENCE OF DEHYDRATASE

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Previously we described a new form of human hyperphenylalaninemia characterized by the formation of 7-substituted pterins.We present evidence strongly suggesting that the 7-substituted pterins are formed by rearrangement of 6-substituted pterins. This rearrangement occurs during the phenylalanine hydroxylase reaction cycle which normally involves the enzymes phenylalanine hydroxylase, pterin-4a-OH-dehydratase, and q-dihydropterin reductase, specifically in the absence of dehydratase activity. We conclude that formation of 7-substituted pterins in humans is a consequence of an absence of dehydratase activity, which might result from a genetic defect. A chemical mechanism for this rearrangement is presented. Our results also suggest that tetrahydroneopterin can be a cofactor for the phenylalanine hydroxylase system *in vivo*.

Our recent detection of 7-substituted pterins (3,4) in some patients with hyperphenylalaninemia suggests the existence of an inborn error of metabolism distinct from the known types of atypical phenylketonuria (PKU) (1). Specifically, L-primapterin and D- or L-anapterin (2,3,4) have been detected in trace amounts in urine of normal individuals, while L-primapterin has been detected in much greater amounts in these atypical PKU patients (biopterin:primapterin ratio ~1:1). In two patients, investigated with primapterinuria, all known enzymes involved in BH_4 metabolism were normal in peripheral blood cells (5), and their activities *in vitro* were not affected by high concentrations of primapterin or by its tetrahydro form; this

Abbreviations and trivial names: L-primapterin,L-erythro-7-iso-biopterin; D- or L-anapterin, D- or L-erythro-7-iso-neopterin.

indicates that the hyperphenylalaninemia in these patients is not due to the effect of primapterin on BH_4 related enzymes. Although the origin of 7-substituted pterins is still unknown, tetrahydrobiopterin (BH_4) loading leads to an equivalent increase in both biopterin and primapterin in the urine. This suggests that primapterin in these patients is derived from BH_4 through some unknown mechanism. In this paper we present evidence supporting the hypothesis that in patients deficient in the enzyme 4a-carbinolamine dehydratase which functions in the conversion of 4a-OH-tetrahydrobiopterin to q-dihydrobiopterin (6) 7-substituted pterins arise from 6-substituted pterins. A detailed chemical mechanism which may explain the conversion is also discussed.

MATERIALS AND METHODS

Materials. Pteridine standards were purchased from Dr. B. Schircks (Jona, Switzerland) and as a gift from Prof. M. Viscontini (University, Zurich). The respective tetrahydro compounds were prepared in our laboratory by catalytic hydrogenation. The dehydratase-free rat liver phenylalanine hydroxylase was purified according to the method of Shiman (7), followed by two further purification steps: ion-exchange chromatography (10 ml DEAE Toyoaperl 650 M) and gel filtration (Sephacryl S-200 HR, column: 1.6 x 100 cm). The activity of the enzyme was assayed fluorometrically (8). Hydroxylase-free dehydratase was obtained according to Parniak (9).

Methods. For the incubation of fecal specimens with 6-BH_4 and 7-BH_4 we used the complex medium VM 10 described by Mitsuoka et al. (10). To the oxygen-free medium, which already contained the bacteria, the pterins were added according to Mettler et al. (11). After incubating the pterins with the medium for different periods of time at 37°C, they were measured by HPLC as described previously (12). For the chemical assay system we used the method of Bobst and Viscontini (13). With the HPLC system tuned for blue fluorescent compounds the pterins were measured after MnO_2 oxidation under acidic conditions. The absence of dehydratase in the rat phenylalanine hydroxylase preparation was shown using the UV assay of Lazarus et al. (6). For the incubation experiments with dehydratase-free rat hydroxylase we incubated 0.1M Tris-HCl, pH 8.45, 1mM L-pheny-lalanine, 0.35 U phenylalanine hydroxylase, 30 μM BH_4 or alternatively D-tetrahydro-neopterin. The mixture was incubated for 45 minutes at 25°C. For regenerating the oxidized pterins formed during the hydroxylation, we added 0.35 U DHPR/ 2 mM NADH and 10 mM dithiothreitol to again reduce the pterins. The organic acid profile was obtained by GC (14).

RESULTS AND DISCUSSION

Theoretically, 7-substituted pterins, including primapterin, can be formed from 6-substituted pterins via several routes, which will be discussed briefly along with experimental evidence in their favor or disfavor.

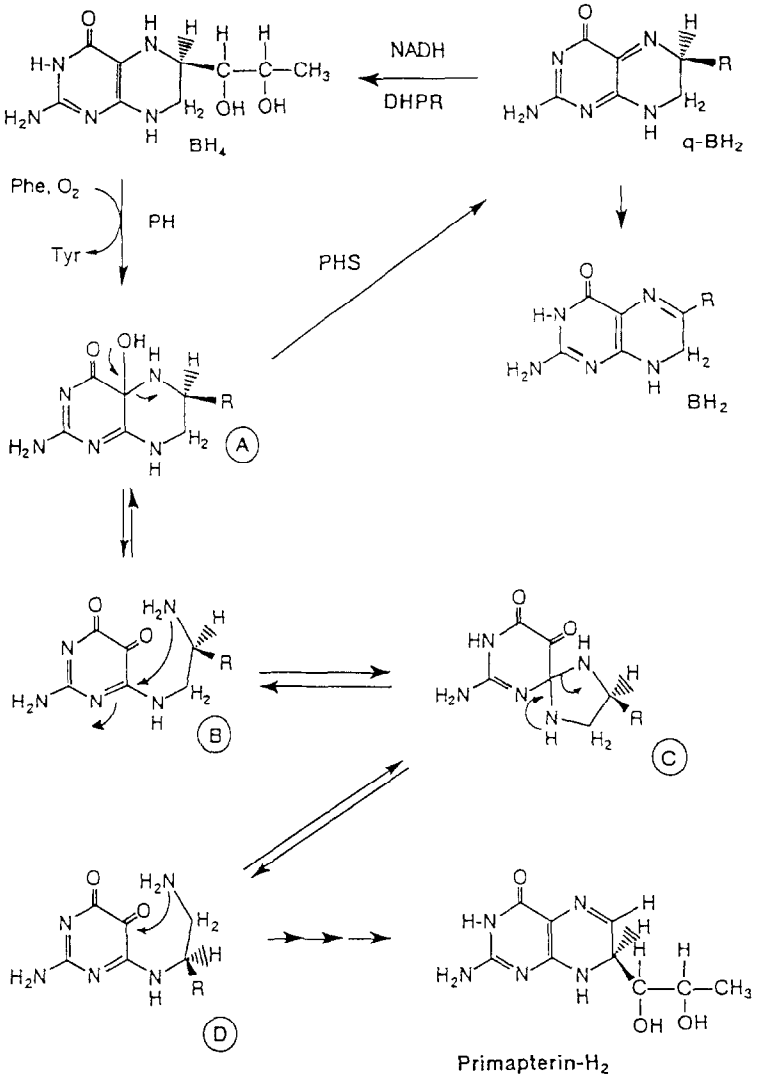
The addition of a dihydroxypropyl side chain to an unsubstituted pterin appears unlikely since this would require the bioavailability of the side chain fragment having the correct stereochemistry and a specific system catalyzing its addition. However, it is conceivable,

that the occurrence of elevated levels of some aliphatic metabolites might lead to such a reaction. We have studied the occurrence of organic acids and keto acids in patients with primapterinuria and found their profile not to differ significantly from that of controls. While this does not conclusively excludes the possibility of an intermolecular addition of the side chain by such a mechanism, it supports the concept that this is highly improbable.

The possibility that the side chain migrates from the 6 to the 7 position and is catalyzed by an enzyme of the gut bacterial flora was also considered since isomerizations such as that of the side chain of tyrosine metabolites from the p- to the o-position are known to occur (11). In incubations of feces from a control and from a patient suffering from lamblia infection with 6-BH_4 and with tetrahydroprimapterin under anaerobic condition 6-BH_4 was completely metabolized to non-fluorescent compounds whereas 7-BH_4 was unchanged. This is in agreement with our observation that in the patient with primapterinuria about 8 times more primapterin than biopterin was detected (data not shown). During incubation of BH_4 under these conditions no conversion of BH_4 to primapterin was observed. Treatment of the primapterinuric patient with high doses of neomycin did not modify the profile in contrast with the ability of neomycin to completely block isomerization of the tyrosine side chain (11).

Lazarus and coworkers (6) have shown that the phenylalanine hydroxylase stimulating protein discovered by Kaufman (15) is a 4a-carbinolamine dehydratase. During the phenylalanine hydroxylase reaction, BH_4 is oxidized to the 4a-carbinolamine intermediate, which is converted by dehydratase to quinonoid-BH_2 as shown in Scheme 1. This dehydration can also proceed nonenzymatically, although at a much slower rate (16). If the dehydration catalysis was impaired, one would expect an accumulation of the carbinolamine in systems catalyzing the hydroxylation of phenylalanine.

Argentini and Viscontini demonstrated that oxidation of BH_4 under physiological conditions might yield iminoalloxan (17) and postulated that this proceeds via 4a-carbinolamine. A dehydratase deficiency causing an accumulation of the 4a-carbinolamine might thus lead to formation of the iminoalloxan and conceivably to a 1,2-diamino-3,4-dihydroxy-pentane residue. Condensation of these two entities in a reverse reaction might form either a 6- or a 7-substituted pterin. Using the chemical assay system (13), we did not get any evidence for such a process. This pathway suffers from the drawback that such a condensation under physiological conditions might not be straightforward, and that both the iminoalloxan and the C_5 residue might undergo decay. This would support the expectation that in contrast to the physiological findings (4)



Scheme 1.

Mechanism proposed to lead to formation of 7-substituted pterins, in the present case primapterin from tetrahydrobiopterin during the phenylalanine hydroxylase reaction. The loop on top of the scheme includes BH_4, the carbinolamine (A), and quinonoid dihydrobiopterin (q-BH_2) as well as the enzymes phenylalanine hydroxylase (PH), the carbinolamine dehydratase (PHS, or phenylalanine hydroxylase-stimulating factor), and dihydropteridine reductase (DHPR). The lower part of the scheme encompasses the steps which are proposed to lead to formation of the 7-substituted pterins. Cyclization of (D) to form primapterin would lead to a carbinolamine analogous to (A), dehydration of this species would lead to quinonoid dihydroprimapterin (analogous to q-BH_2), and tautomerization of the latter forms the dihydro primapterin shown.

formation of 7-substituted pterins should be, at best, a minor side reaction. Further more, in the *in vitro* assay the presence of a scavenger of vicinal-dicarbonyl structures should affect the yield of 7-pterins. When the phenylalanine hydroxylase reaction was carried out in the presence of equimolar and excess amounts of o-phenylenediamine, the relative yield of primapterin was unaffected. This experiment does not support the

occurrence of free alloxan type intermediates. A different route is depicted in Scheme 1, details of which will be discussed below.

In order to check the validity of the hypothesis involving a dehydratase deficiency and the accumulation of the carbinolamine intermediate, we carefully analyzed the pterins formed in the phenylalanine hydroxylase reaction under varying conditions. The results are exemplified by the analytical HPLC profiles shown in Figure 1. The peak identification was obtained by spiking the samples with reference substances and by coelution with pterin standards. It is clear that in the presence of dehydratase no primapterin is formed, while in its absence it can be identified in relatively small amounts, e.g., under our assay conditions primapterin is ~4% of total biopterin.

It is likely that *in vivo* the 7-pterins will accumulate since they are not as good a substrate for phenylalanine hydroxylase as the 6-pterins (data not shown). The finding of anapterin in patients (3) has been puzzling, but it can be rationalized in view of the present experiments which strongly suggest that at least under the particular conditions of the patients suffering from anapterinuria neopterin at its various redox states can serve as cofactor for the phenylalanine hydroxylase reaction. When incubating tetrahydroneopterin with dehydratase-free hydroxylase only a very small amount of anapterin is formed (~0.4% of total neopterin). Addition of dehydratase during incubation removed only half of the anapterin formed.

In view of the highly unusual course of the isomerization reaction in question, a few comments seem appropriate: The dehydratation of a 4a-carbinolamine (structure (A),

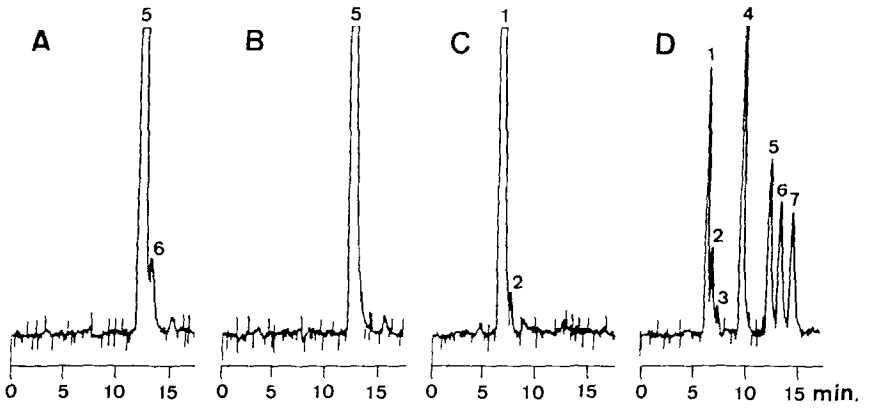


Figure 1.
HPLC chromatograms: (A) incubation of BH_4 without dehydratase; (B) incubation of BH_4 with dehydratase; (C) incubation with tetrahydroneopterin without dehydratase; (D) reference chromatogram; 1: neopterin; 2: anapterin; 3: monapterin; 4: isoxanthopterin; 5: biopterin; 6: primapterin; 7: threo-biopterin.

Scheme 1), while a normal chemical process (16), is not rapid and consequently species (A) has a rather long life time. For the reactions depicted in the scheme, the pK for protonation of the carbinolamine N(5)-H might be crucial: An OH function in position α of an alcohol is known to lower its pK by 3-4 units (18), and one might expect a similar effect on an amine function. This would locate the pK of N(5)-H around neutrality. A relevant equilibrium concentration of N(5) protonated carbinolamine might induce (reversible) ring opening to form (B). Attack of the N(5) amino group of (B) at the imino function, i.e., at position 6 of the pyrimidine ring, forms the spiro structure (C); this reaction is likely to compete with an attack at position 5 (back formation of (A)) . (C) might thus exist in equilibrium with species (B) and (D). The reaction of the latter to form dihydroprimapterin via the corresponding carbinolamine, elimination of water to form the quinonoid dihydroprimapterin, and tautomery do not pose particular chemical problems.

Along this line anything prolonging the life time of the carbinolamine (A), such as the absence of a dehydratase, would favor any of these rearrangement reactions. Final proof that our proposal is correct will have to await measurement of dehydratase activity in liver biopsy material from primapterinuric patients.

Finally, our results suggest that an important corollary role of the dehydratase is the prevention of formation of 7-pterins.

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