Reexamination of Reduced Pteridine Cofactors Behaviour by Proton Nuclear Magnetic Resonance Spectroscopy and Mass Spectrometry

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In aqueous solutions, the autoxidation by air of 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine, a hydroxylase cofactor, leads to the corresponding 7,8-dihydro derivate. Oxidation by hydrogen peroxide and Horseradish peroxidase does not give a stable quinonoid form as previously claimed but affords two metabolic products. Kinetics of the two pathways and structures of the different final compounds were determined by ^1H nmr and mass spectrometry.

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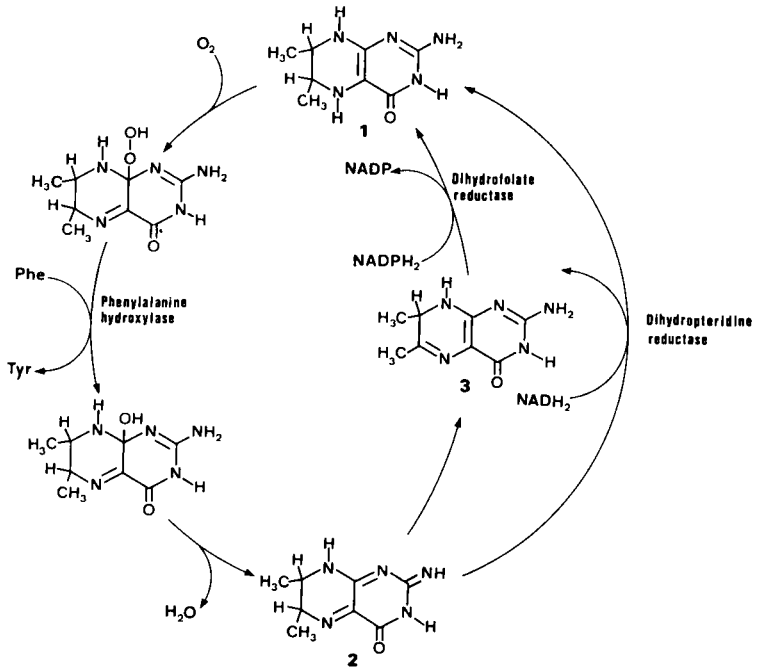
Tetrahydropteridines are known to act as cofactors in the enzymatically catalyzed hydroxylation of phenylalanine to tyrosine (1,2). Tetrahydrobiopterine is the natural cofactor of this reaction (3,4). However, various synthetic tetrahydropteridines exhibit a similar activity (2,5,6,7). Such is the case for 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine **1** (Scheme I). The enzymatic hydroxylation was shown to involve the following two enzymes (1,8): (a) phenylalanine hydroxylase, which catalyses the conversion of phenylalanine to tyrosine (the tetrahydropteridine is subsequently oxidized to an "active form" of dihydropteridine); and (b) dihydropteridine reductase, which catalyses the reduction of this intermediate back to the corresponding initial tetra-hydropteridine, in the presence of NADH or NADPH (9,10).

According to Hemmerich (11) and Kaufman (12), the key intermediate involved in the oxidation-reduction cyclic sequence of pteridine cofactors is a quinonoid derivate **2**. When there is no reductant, this unstable cofactor is converted to a more stable isomeric form, presented as a 7,8-dihydropteridine **3**, inactive towards dihydropteridine reductase. However, **3** can be reduced to **1** by dihydrofolate reductase (4,7,9,13).

Other authors have suggested the formation of various other intermediates (14,15,16,17). Mager, in particular, has proposed the intervention of a hydroperoxide which would be responsible for the aromatic hydroxylation of substrates such as phenylalanine. It is claimed that "solutions of the active dihydropteridine cofactor (quinonoid form **2**) were prepared by oxidation of the corresponding tetrahydropteridines. This synthesis was car-



Scheme I

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ried out by the use of hydrogen peroxide and peroxidase" (10,11,12,13).

We have reinvestigated the study of the evolution of the tetrahydropteridine cofactor **1** under the previously described experimental conditions. We have done so, firstly by autoxidation in air, and secondly by oxidation in the presence of hydrogen peroxide-peroxidase. In the first case, proton nuclear magnetic resonance spectroscopy has enabled us to determine unambiguously the spatial structure of **1** and **3**. For the second case, in the conditions described by Nielsen (13), with the hydrogen peroxide-peroxidase system, two compounds clearly lacking the quinonoid structure **2** were isolated by the authors. Reported here are possible mechanisms for the two different oxidation pathways.

Autoxidation by Air.

The uv absorption spectra recorded at different stages during the aerobic oxidation of **1** in aqueous solution are very similar to those described by Kaufman (18), Nielsen (13) and Mager (15). An intermediate curve with a higher absorbance at 305 nm could correspond to the quinonoid dihydropteridine **2**.

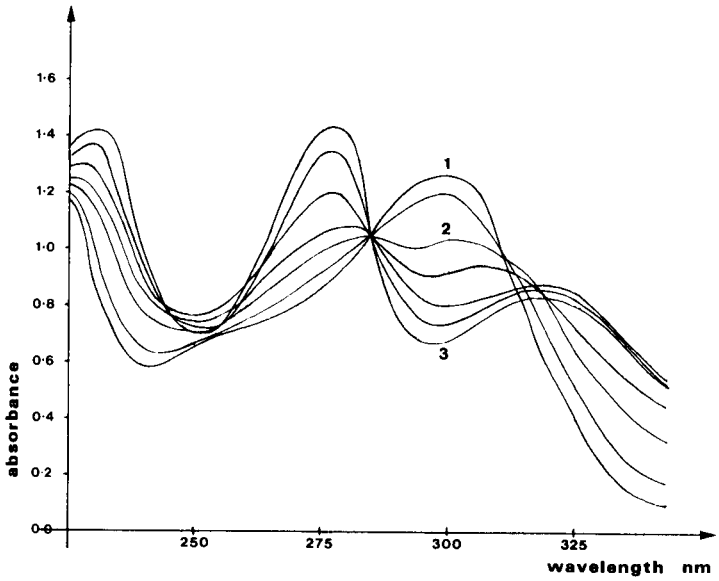


Figure 1. Uv absorbance spectra of tetrahydropteridine autoxidation kinetics (see Scheme I for derivative numbers).

Using nmr spectroscopy, the kinetics of the autoxidation of **1** have been studied under the same conditions. It is interesting to note the stereochemistry of the tetrahydropteridines during the first stage.

It has been both theoretically predicted (19,20) and experimentally confirmed (21,22,23) that the spin-spin coupling of protons on adjacent carbon atoms is a function of the dihedral angle describing the degree of rotation about the bond joining the carbon atoms. For **1**, the $J_{H_a-H_b}$ value corresponds to a dihedral angle of 50° or 130°. If the value of the dihedral angle is α = 130°, the pyrazine ring of **1** exists in a boat conformation (**1a**). If α = 50°, the cycle exists in a twist conformation (**1b**).

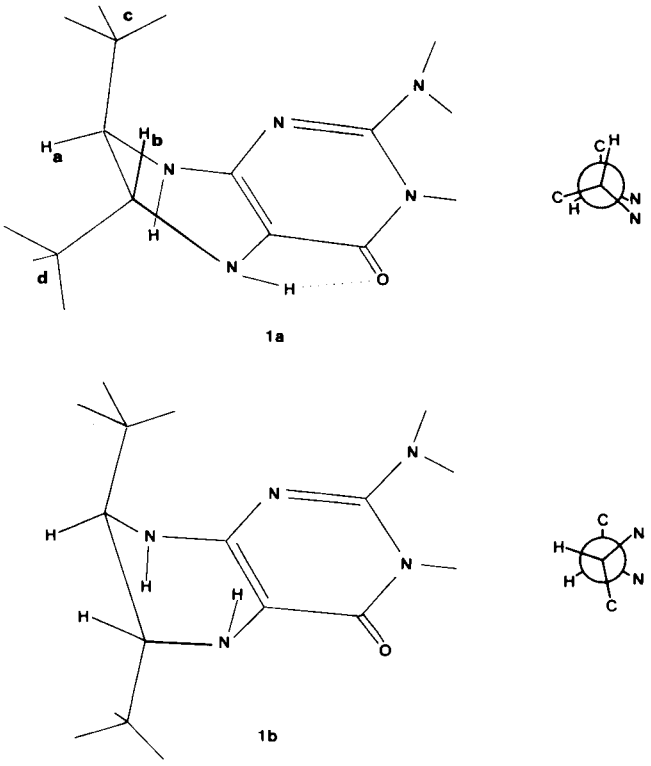


Figure 2. Possible stereospecific structures of **1**. The **1a** form appears to be more stable in that this conformation permits, in addition, the establishment of relatively strong hydrogen bonding.

The oxidation evolves slowly in the nmr tube where contact with air is not favoured. The spectral data of **3** demonstrates the disappearance of proton b. The corresponding structure does confirm the formula proposed by Kaufman. Intermediate spectra were recorded, however they are not included here for lack of further information. In particular no quinonimid structure was observed.

Oxidation in the Presence of Hydrogen Peroxide-Peroxidase.

Figure 3 represents the kinetics of oxidation in a hydrogen peroxide-peroxidase medium. The uv spectra are the same as those of Nielsen (13).

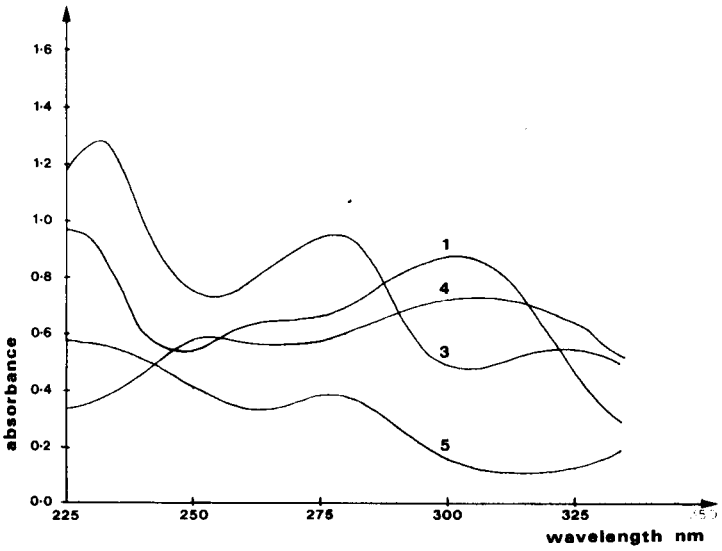


Figure 3. Uv absorbance spectra of derivates **1**, **3**, **4** and **5** during the oxidation of **1** by hydrogen peroxide and Horseradish peroxidase.

The oxidation is fast and this is clearly demonstrated on the nmr spectra; **1** evolves quickly to the stable compound **4**, the spectrum of which has no similarity to the spectra described above.

The bridgehead C-atoms have an sp^3 hybridization and the oxygen atom attracts the hydrogen atom of the N-H group to establish a hydrogen bond. In this stabilized form, the methyl group and the proton e (Figure 4) is influenced by the carbonyl anisotropic cone. This may justify the demonstrated deshielding.

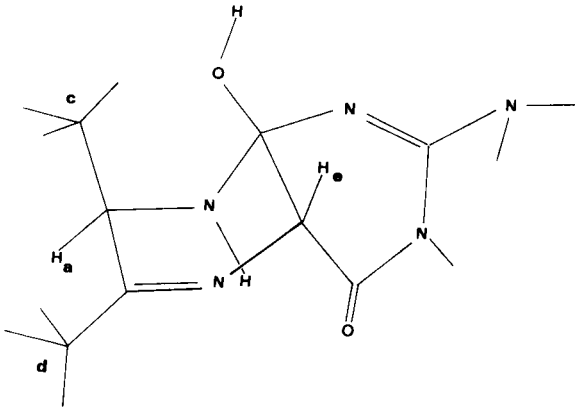
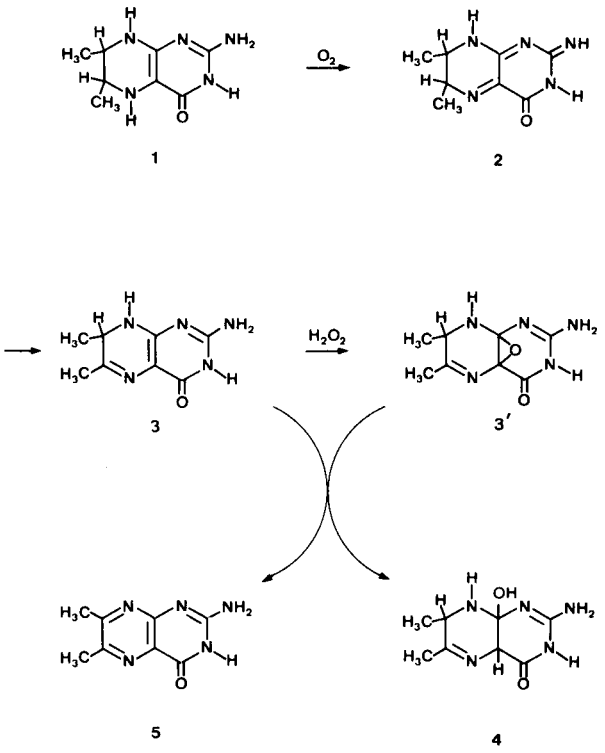


Figure 4. Spatial structure of **4**.

During the oxidation, a second metabolic product was found to precipitate in the medium. The compound was collected and identified as 2-amino-6,7-dimethyl-4-(3*H*)-pteridinone (**5**) by mass spectrometry.

The authors propose here a chemical mechanism which may explain the concomitant formation of **4** and **5** (Scheme II).



After an initial oxidation to 7,8-dihydropteridine **3**, an epoxide **3′** is formed by the addition of hydrogen peroxide on the double hinge-bond, as described in similar cases (24,25). It is known that in reducing media, oxygen-bridges of oxiranes are broken yielding alcohols (26,27,28). The reducing form of **3** is favourable to this opening. An intermolecular mechanism could be postulated (Scheme II).

Discussion.

In order to prove the existence of the quinonoid dihydroform, the tetrahydropteridine oxidation kinetics with both air and a hydrogen peroxide-peroxidase system were studied. This may possibly be the key intermediate cofactor for dihydropteridine reductase.

The derivative **3** obtained by oxidation with air is the 7,8-dihydropteridine as previously reported. However, the authors can affirm that in the hydrogen peroxide-peroxidase medium, the quinonoid form which could occur, is not more "stabilized" than by the autoxidation process. Under these experimental conditions, the tetrahydropteridine would rapidly evolve to a soluble hydroxy compound not previously mentioned up to the present and to a pteridine which is completely oxidized.

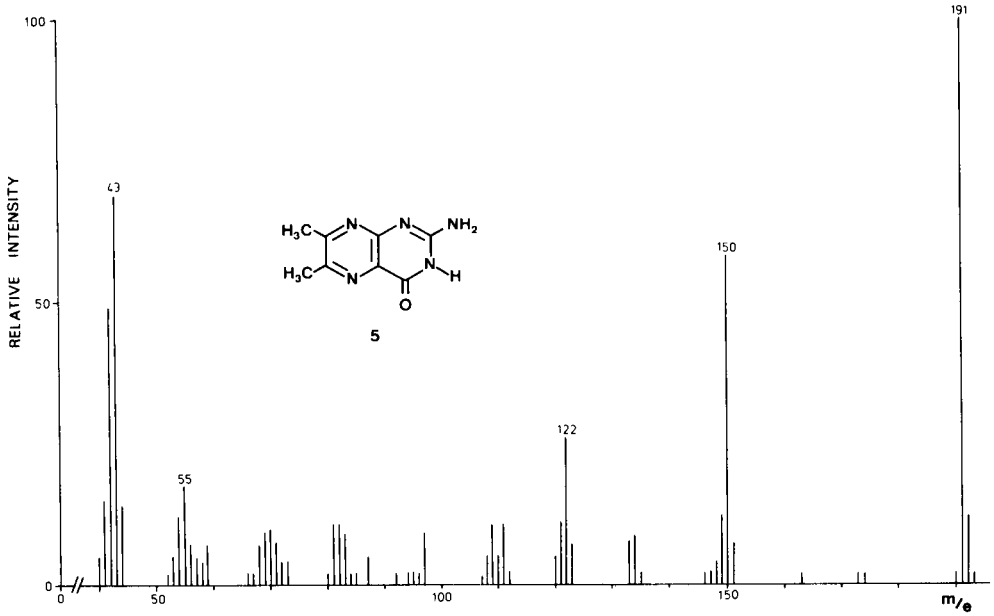


Figure 5. Mass spectrum of **5**, precipitate occurring during the oxidation of **1** by hydrogen peroxide-Horseradish peroxidase. It is identical with the spectrum of 2-amino-6,7-dimethyl-4-(3*H*)pteridinone reported by Williams and Ayling (29).

It is obvious that the uv curve of Figure 3 corresponds to these compounds rather than to the quinonoid form as it was proposed by Nielsen. In additon, the compound **3** was submitted to the further action of the hydrogen peroxide-peroxidase system and the final products **4** and **5** were also isolated, confirming the mechanism shown in Scheme II.

In both reactions no quinonoid structure was observed by nmr spectroscopy, though the uv specta recorded under the same experimental conditions were similar to those described by Kaufman and Nielsen. Nevertheless, the implication of a quinonoid form cannot be dismissed. It could occur from the outset of the reaction and be rapidly converted into the dihydroform **3**. Conversion to the aromatic compound **5** and the hydroxyderivative **4** (with hydrogen peroxide and peroxidase) in the absence of a reductant system (dihydropteridine reductase) may then occur.

EXPERIMENTAL

2-Amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine hydrochloride was obtained from Calbiochem and Horseradish peroxidase from Sigma. Spectrophotometric measurements were made with a Unicam SP 800 UV Spectrophotometer. ^{1}H Nmr spectra were recorded from a JEOL-60 instrument. Mass spectra were obtained from a AEI MS 30 spectrometer.

Autoxidation of Aqueous Solutions of **1**.

An aqueous solution of tetrahydropteridine hydrochloride 0.1m*M* with a Tris-hydrochloric acid buffer (0.1*M*, *p*H 7.1) or neutralized by sodium carbonate was left at room temperature in equilibrium with the air for 2 hours. The kinetics of the oxidation were followed by uv spectrophotometry. Under the same conditions with tetrahydropteridine hydrochloride neutralized by sodium carbonate, the kinetics of oxidation were studied by ^{1}H nmr spectroscopy in deuterium oxide with DSS as internal standard.

Tetrahydropteridine **1**.

This compound had ^{1}H nmr: Ha (m) δ 3.20, Hb (m) 3.60, J_{HaHb} = 3 Hz, CH_3c and CH_3d (d) 1.08, $J_{H\text{-}CH_3}$ = 6.8 Hz.

Dihydropteridine **3**.

This compound had ^{1}H nmr: Ha (q) δ 2.98, CH_3c (d) 1.25, J_{HaCH_3c} = 6.8 Hz, CH_3d (s) 0.75.

Oxidation in the presence of Hydrogen Peroxide and Peroxidase.

The procedure utilized was that described by Nielsen (6). The absorbance spectra were measured immediately after the addition of Hydrogen Peroxide (50m*M*) and Horseradish peroxidase (20 μl./ml.) to an aqueous solution of **1** (0.1m*M*) in a Tris-hydrochloric acid buffer (0.1*M*). By the same process, the evolution of **1**, neutralized by sodium carbonate, in the presence of hydrogen peroxide and peroxidase was followed by ^{1}H nmr. The 60 MHz ^{1}H nmr spectrum of **4** in deuterium oxide (δ ppm) with DSS as internal standard revealed: Ha (q) δ 2.95, CH_3c (d) 1.33, J_{HaCH_3c} = 6.8 Hz, CH_3d (s) 1.60, He (s) 4.00.

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