REACTION OF Cu^{2+} AND Fe^{3+} WITH TETRAHYDROPTERIDINES

D. J. Vonderschmitt* and K. G. Scrimgeour

Department of Biochemistry
Scripps Clinic and Research Foundation
La Jolla, California 92038

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This communication describes several novel reactions that have been observed during a study of the interaction of tetrahydropteridines with transition metal ions under anaerobic conditions. By the use of polarographic methods, similar to those previously described for pteridines by Asahi (1964) and Komenda and Laskafeld (1962), we have demonstrated that Cu^{2+} forms a stable complex with tetrahydropteridines, and that Fe^{3+} oxidizes tetrahydropteridines via a complex series of reactions in which quinonoid- and 7, 8-dihydropteridine intermediates are formed. The latter reaction may serve as a useful model for the mechanism of the tetrahydropteridine- and metal ion-dependent enzymatic hydroxylation of phenylalanine (Kaufman, 1964; Guroff and Ito, 1965).

METHODS

2-Amino-4-hydroxytetrahydropteridine (tetrahydropterin) sulfate was prepared from the oxidized pterin (K and K Laboratories) by catalytic hydrogenation in trifluoroacetic acid (Bobst and Viscontini, 1966). Tetrahydrofolate was prepared by the method of Hatefi et al. (1960); excess acetic acid was removed at 80° under high vacuum using solid NaOH as adsorbent. Polarography of the reaction mixtures was performed under purified nitrogen using a Heath Model EUW-401 polarography system equipped with a Metrohm E354S synchronous mercury drop controller and a Ag/AgCl reference electrode. Fe^{3+} was estimated

* Present address: Universite de Neuchatel, Institut de Chimie, Neuchatel, Switzerland.

by adding excess sulfosalicylic acid or thiocyanate and comparing the absorbance at 480 mμ to standard solutions of $FeNH_4(SO_4)_2$. Fe^{2+} was determined as the o-phenanthroline complex by its absorbance at 512 mμ.

RESULTS

Interaction of Copper with Tetrahydropteridines.

Cu^{2+} ($CuSO_4$) reacted at pH values above 4 with tetrahydropterin to yield a transient, purple complex, followed almost immediately by a brown precipitate. Formation of the complex could be determined by observing the decrease at + 0.1 volts in diffusion current of the anodic oxidation wave of tetrahydropterin at pH 6.0 (0.12 M 2-(N-morpholino)-ethanesulfonic acid buffer; 0.25 N $NaClO_4$). No formation of dihydropterin or Cu^{+} could be detected. Tetrahydrofolate also formed a complex with Cu^{2+}, and was more suitable for these studies because it did not precipitate until excess copper reacted with the glutamate carboxyl groups. The tetrahydrofolate-copper complex had an absorption maximum at 278 mμ, and a very broad band in the visible region. The extent of reaction of tetrahydrofolate with Cu^{2+} was directly proportional to the concentration of the metal ion up to the point of precipitation, indicating that the binding of Cu^{2+} by the reduced pteridine moiety was rather strong. Amperometric titration of tetrahydropterin (and of tetrahydrofolate) with Cu^{2+} indicated that more than 1 μatom of Cu^{2+} reacted with each μmole of reduced pteridine, but these studies were limited by the insolubility of the complexes.

Interaction of Iron with Tetrahydropteridines.

The interaction of Fe^{3+} (as $FeNH_4(SO_4)_2$) with tetrahydropterin was readily measured at pH 3.2 in formate or chloroacetate buffer. At this pH, Fe^{3+} was soluble without the addition of a complexing agent.

In order to examine the stoichiometry of the overall reaction, tetrahydropterin was treated with increasing amounts of Fe^{3+}, and polarograms were recorded in each instance. Polarographic waves were identified by comparison to those of the individual components: tetrahydropterin, $E_{1/2}$ = + 0.2 volts (anodic); oxidized pterin, $E_{1/2}$ = - 0.4 volts (cathodic); and 7,8-dihydropterin, $E_{1/2}$ = - 0.9 volts (cathodic). Excess Fe^{3+} has a polarographic wave at $E_{1/2}$ = + 0.1 volts (mixed wave).

Figure 1 demonstrates the stoichiometry observed from polarographic studies of Fe^{3+}-tetrahydropterin interaction. For this experiment, it was

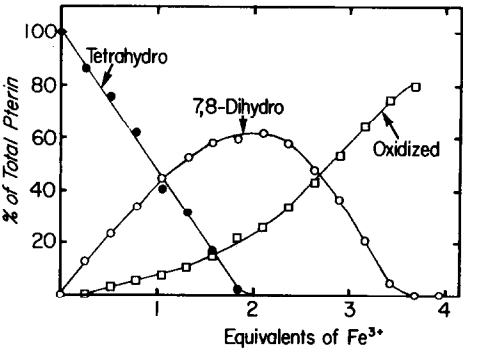

Fig. 1 Oxidation of tetrahydropterin by $FeNH_4(SO_4)_2$

Tetrahydropterin (56 μmoles) was dissolved at 23° in 20 ml. of a deaerated solution containing formate buffer, pH 3.2 (5.0 mmoles) and $NaClO_4$ (5.0 mmoles). Deaerated 0.1 N $FeNH_4(SO_4)_2$ was added as indicated (1 equivalent of Fe^{3+} = 0.56 ml.). After completion of reaction in each instance, the amounts of pteridine in each oxidation level were estimated polarographically: tetrahydropterin from the change in diffusion current (Δi_d) at + 0.24 volts, 7,8-dihydropterin from Δi_d (- 0.8 to 1.04 volts), and oxidized pterin from Δi_d (- 0.16 to 0.8 volts). All i_d values were corrected for dilution.

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assumed that tetrahydropterin, 7,8-dihydropterin, and oxidized pterin had identical diffusion coefficients. After each increment of Fe^{3+} had been added, the reaction was allowed to reach completion (ca. 20 minutes at 23°), and then the concentration of each oxidation level of pterin was determined polarographically. During the later stages of the experiment, some of the oxidized pterin precipitated causing the total pterin observed polarographically to be less than 100%. Excess Fe^{3+} appeared only after all the tetrahydropterin and 7,8-dihydropterin had been oxidized. In a separate experiment, it was demonstrated that, after completion of the reaction, the stoichiometry corresponded to 4 μatoms of Fe^{3+} reduced per μmole of tetrahydropterin oxidized (e.g., oxidation of 0.15 μmoles of tetrahydropterin produced 0.58 μatoms of Fe^{2+}). At neutral pH (in the presence of either 0.12 M EDTA or 0.03 M oxalate to prevent hydrolysis of Fe^{3+}), tetra-

hydropterin was still oxidized by Fe^{3+}, but the subsequent oxidation of dihydropterin was much slower than at pH 3.2.

The above studies indicated that at pH 3.2 tetrahydropterin was completely oxidized to pterin, with 7, 8-dihydropterin as an intermediate. The equation for the overall reaction is:

$$4\ Fe^{3+} + \text{Tetrahydropterin} \longrightarrow 4\ Fe^{2+} + \text{Oxidized pterin} \qquad (1)$$

By repeating these experiments, two distinct steps could be observed in the formation of 7, 8-dihydropterin from interaction of Fe^{3+} with tetrahydropterin. First, there was the rapid formation of a compound (λ_{max} at about 333 mμ) which was also characterized by a cathodic polarographic wave at + 0.1 volts. The spectrum and polarographic wave of the intermediate were similar to those of a "quinonoid"-dihydropterin, which we have prepared by oxidation of tetrahydropterin with indophenol according to the method of Kaufman (1959). The reduction of Fe^{3+} to Fe^{2+} occurred within the first few seconds after addition of Fe^{3+} to tetrahydropterin. The required stoichiometry between the reduction of 2 equivalents of Fe^{3+} and the formation of 1 equivalent of quinonoid-dihydropterin has been established by the method of continuous variation (Rossotti and Rossotti, 1961), using the absorbancy at 333 mμ as a measure of the concentration of the intermediate quinonoid-dihydropterin.

The second step in the sequence was the relatively slow conversion of the quinonoid intermediate to 7, 8-dihydropterin. The course of this reaction, followed by polarography, obeyed first-order kinetics with respect to disappearance of the quinonoid-dihydropterin. Tautomerism of the quinonoid form to the 7, 8-form occurred more rapidly as the pH was increased.

7, 8-Dihydropterin probably is not oxidized by Fe^{3+}, but undergoes further reaction with the quinonoid-dihydropterin to give tetrahydropterin and oxidized pterin. The tetrahydropterin formed would then be oxidized to the quinonoid-dihydropterin, and the latter would act as a catalyst for the oxidation of 7, 8-dihydropterin by Fe^{3+}. In support of this type of process, we have observed that the quinonoid form, in the absence of excess oxidant, yields 7, 8-dihydropterin, oxidized pterin, and tetrahydropterin.

DISCUSSION

Both Cu^{2+} and Fe^{3+} interacted with tetrahydropteridines, although there is a difference in their reactions. Cu^{2+} and tetrahydrofolate form a stable purple complex similar to that observed between 5-methyltetrahydrofolate and Cu^{2+} (R. R. Schmidt and D. J. Vonderschmitt, unpublished results). The spectra of both these purple products suggest that they might be stable radical complexes, resembling that formed from peroxidation of a 5-methyltetrahydropterin (Ehrenberg et al., 1967).

In the case of Fe^{3+}, there is rapid oxidation of tetrahydropterin and a simultaneous reduction of Fe^{3+} to Fe^{2+} (reactions 2-4).

$$\text{Tetrahydropterin} + 2\ Fe^{3+} \xrightarrow{\text{fast}} \text{Quinonoid-dihydropterin} + 2\ Fe^{2+} \quad (2)$$

$$\text{Quinonoid-dihydropterin} \longrightarrow \text{7, 8-Dihydropterin} \quad (3)$$

$$\text{Quinonoid-dihydropterin} + \text{7, 8-Dihydropterin} \longrightarrow \text{Tetrahydropterin} + \text{Pterin} \quad (4)$$

A quinonoid-dihydropterin also appears to be the first dihydro isomer produced in the oxidation of tetrahydropteridines by indophenol (Kaufman, 1959) or oxygen (Viscontini and Bobst, 1965), as well as during the enzymatic hydroxylation of phenylalanine (Kaufman, 1964). The polarographic half-wave potentials for the oxidation of tetrahydropterin and for the reduction of quinonoid-dihydropterin are the same. These reactions, therefore, are polarographically reversible. Conversely, the polarographic reduction of 7, 8-dihydropterin to tetrahydropterin, which appears at a much more negative voltage, is polarographically irreversible. The quinonoid-dihydropterin is thus a thermodynamically unstable, but kinetically favored, intermediate in the oxidation of tetrahydropterins. Anaerobic reactions involving indophenol or Fe^{3+} show that oxygen is not essential for formation of the quinonoid isomer.

The oxidation of tetrahydropterin by Fe^{3+} was too rapid to allow the mechanism of reaction (2) to be studied by the present techniques. The trihydropterin radical (Ehrenberg et al., 1967) is very probably an obligatory intermediate in this reaction, but it could not be detected by our methods. It is possible that dissociation of the N-3 proton of

the tetrahydropterin (i.e. formation of the 4-phenolate ion at higher pH) would stabilize the trihydropterin-Fe^{2+} radical complex proposed by Ehrenberg et al. It has recently been proposed by Scrimgeour et. al. (1967) that the reduction of folate by reduced ferredoxin also proceeds through a radical intermediate, providing another example of oxido-reduction of the pyrazine ring by one-electron steps.

Many mechanisms have been proposed for the hydroxylation of aromatic substrates by mixed function oxidases (Mason, 1957). For the hydroxylation of phenylalanine, Ehrenberg et al. (1967) have recently postulated a coordinated linkage of Fe^{3+} to the tetrahydropteridine cofactor, followed by the reduction of the Fe^{3+} to Fe^{2+} by the cofactor. Our experiments with the uncomplexed metal ion at low pH values have demonstrated for the first time the reduction of Fe^{3+} by tetrahydropteridines, but the conditions of our experiments differ from those of the enzymatic process with respect to pH, exclusion of oxygen, and coordinative environment of iron. We are currently examining the effects of pH and metal chelation on reaction (2) to see if the oxidation of tetrahydropterin by Fe^{3+} can be stopped at the radical state, and thus allow detection and characterization of an intermediate similar to that proposed by Ehrenberg et al.

Further details of the interactions of metal ions with tetrahydropteridines, as well as the polarographic analyses of the quinonoid-dihydropterin and its isomerization to 7,8-dihydropterin, will be reported in subsequent publications.

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