New pteridine substrates for dihydropteridine reductase and horseradish peroxidase

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The oxidation of 4,5-diaminopyrimidin-6(1*H*)-one, 5,6,7,8-tetrahydropteridin-4(3*H*)-one, its 6-methyl and *cis*-6,7-dimethyl derivatives, and 6-methyl- and *cis*-6,7-dimethyl-5,6,7,8-tetrahydropterins, by horseradish peroxidase/H₂O₂ is enzymic and follows Michaelis–Menten kinetics, and its *K*_m and *k*_{cat} values were determined. This oxidation of 5,6,7,8-tetrahydropterins produces quinonoid dihydropterins of established structure, and they are known to be specific substrates for dihydropteridine reductase. By analogy the peroxidase/H₂O₂ oxidation of the 5,6,7,8-tetrahydropteridin-4(3*H*)-ones should produce similar quinonoid dihydro species. The quinonoid species derived from 5,6,7,8-tetrahydropteridin-4(3*H*)-one and its 6-methyl and *cis*-6,7-dimethyl derivatives are shown to be viable substrates for human brain dihydropteridine reductase, and apparent *K*_m and *V*_{max} values are reported.

INTRODUCTION

Dihydropteridine reductase (EC 1.6.99.7) catalyses the reduction of quinonoid 7,8-dihydro-*L*-erythro-(6*H*)biopterin (1) to 5,6,7,8-tetrahydro-*L*-erythro-(3*H*)biopterin (4) in the presence of NADH. Simple alkylpterins, e.g. compounds (2) and (3), are effective substrates and can replace the natural compound (1) in this enzymic reaction (Armarego *et al.*, 1984). In Nature quinonoid dihydro-

biopterin is produced from the tetrahydro derivative (4) during the hydroxylation of phenylalanine, tyrosine and tryptophan by the enzymes phenylalanine hydroxylase (EC 1.14.16.1), tyrosine hydroxylase (EC 1.14.16.2) and tryptophan hydroxylase (EC 1.14.16.4) in the presence of O₂ to form tyrosine, 3,4-dihydroxyphenylalanine (dopa) and 5-hydroxytryptophan respectively (Kaufman & Fisher, 1974). Quinonoid dihydropterins can also be formed quantitatively from the respective 5,6,7,8-

(1) R¹ = (2'-*R*, 3'-*S*)-CHOHCHOHMe
R² = H

(2) R¹ = Me, R² = H

(3) R¹ = R² = Me

(4) R¹ = (2'-*R*, 3'-*S*)-CHOHCHOHMe
R² = H

(5) R¹ = Me, R² = H

(6) R¹ = R² = Me

(7)

(8) R¹ = R² = R³ = R⁴ = H

(9) R¹ = Me, R² = R³ = R⁴ = H

(10) R¹ = R³ = Me, R² = R⁴ = H

(11) R¹ = R² = R³ = R⁴ = H

(11a) R¹ = R² = R³ = R⁴ = ²H

(12) R¹ = Me, R² = R³ = R⁴ = H

(12a) R¹ = Me, R² = R⁴ = ²H, R³ = H

(13) R¹ = R³ = Me, R² = R⁴ = H

(13a) R¹ = R³ = Me, R² = R⁴ = ²H

Abbreviation used: pterin is the generic name for 2-aminopteridin-4(3*H*)-one.

tetrahydropterins at physiological pH by oxidation with molecular O_2 (Kaufman, 1961; Viscontini & Bobst, 1965; Mager, 1975; Pearson & Blair, 1975; Armarego & Schou, 1978), Br_2 (Lazarus *et al.*, 1982), Fe^{3+} (Archer *et al.*, 1972), $K_3Fe(CN)_6$, 2,6-dichlorophenolindophenol (Kaufman, 1961; Nielsen *et al.*, 1969; Archer *et al.*, 1972), ferricytochrome *c* (Hasegawa *et al.*, 1978) and peroxidase in the presence of H_2O_2 (Nielsen *et al.*, 1969) or O_2 (Armarego *et al.*, 1983a). The most commonly used method for generating quinonoid dihydropterins rapidly and quantitatively for assaying dihydropteridine reductase is the Nielsen *et al.* (1969) peroxidase/ H_2O_2 method (Craine *et al.*, 1972; Chauvin *et al.*, 1979; Firgaira *et al.*, 1979; Armarego *et al.*, 1983a,b, 1984; Shen, 1984). The structures of the quinonoid dihydropterins produced by the different oxidants and the aromatic amino acid hydroxylases are the same, and have been the subject of several studies. Kaufman (1964) proposed the *para*-quinonoid structure (7) after a suggestion by Hemmerich (1964), and presented good evidence for the pyrazine moiety of structure (7). This has subsequently been confirmed by several other methods (cf. Armarego *et al.*, 1984). However, the exact tautomeric structure of the pyrimidine ring has been questioned and kinetic evidence presented to show that the tautomer with the endocyclic double bond [i.e. between C-2 and N-3, as in structures (1), (2) and (3)] was the reactive tautomer (Armarego & Waring, 1982, 1983b). A study of the chemical shifts of the ^{15}N nuclear magnetic resonances upon oxidizing the tetrahydropterins (6) to the quinonoid dihydropterins labelled with this isotope revealed that in aqueous solution at physiological pH the predominant tautomer is the one with the endocyclic double bond in the pyrimidine ring (3) (Benkovic *et al.*, 1985). We argued that if the quinonoid dihydropterins that reacts as a substrate for dihydropteridine reductase has the tautomeric structure (1)–(3), then the quinonoid dihydropteridines (8)–(10) derived from the 5,6,7,8-tetrahydropteridines (11), (12) and (13), which do not have the 2-amino group, should also be substrates for this enzyme. We have studied the tetrahydropteridines (11)–(13), and report that their products of oxidation by peroxidase/ H_2O_2 are substrates for dihydropteridine reductase and show Michaelis–Menten kinetics from which satisfactory K_m and k_{cat} values were determined. Further, in order to demonstrate that the quinonoid dihydropteridines (8)–(10) may have tautomeric structures similar to the quinonoid dihydropterins (2) and (3), the oxidation of their respective 5,6,7,8-tetrahydro derivatives (11), (12) and (13), and of compounds (5) and (6), by peroxidase/ H_2O_2 was studied. All these compounds were shown to be oxidized enzymically, and their apparent K_m and V_{max} values are reported.

MATERIALS AND METHODS

General methods

Kinetic measurements were performed on a Unicam SP.1800 double-beam spectrometer and Rikadenki model B-281H recorder with 5 mV across the slide wire to produce a maximum pen movement corresponding to 0.1 absorbance unit. For higher sensitivity a Cary 219 instrument adjusted to produce maximum pen movement of 0.01 absorbance unit was used, and accurate

measurements of absorbances for concentration determinations were performed on a Perkin–Elmer Lambda 1 single-beam spectrometer. T.l.c. was run on silica gel 60 F₂₅₄ (Merck) and eluted with butan-1-ol/acetic acid/water (20:3:7, by vol.). The spots were revealed by u.v. light (at 250 and 365 nm) and staining with I_2 vapour.

Tetrahydropteridines

The tetrahydropteridines (11)–(13a) used were analytically pure and their syntheses and spectroscopic properties are reported elsewhere (Armarego *et al.*, 1986).

Dihydropteridine reductase

The enzyme from human brain was purified as described previously (Armarego & Waring, 1983a) but with a freshly prepared naphthoquinone column (Cotton & Jennings, 1978), and gave one band (M_r 26000) on SDS/polyacrylamide-gel electrophoresis without passage through a 5'-AMP-Sepharose column. Protein concentrations were measured with the Bio-Rad Laboratories (Richmond, CA, U.S.A.) standard assay kit and the microassay kit (for concentrations less than 25 μ g/ml), with bovine serum albumin (Bio-Rad Laboratories) as standard. This stock enzyme solution (0.39 mg/ml) was divided into 2 ml portions and frozen in glass vials. These can be thawed and refrozen several times without loss of activity, and the frozen solutions can be stored at below $-10^\circ C$ for more than a year without loss of activity. Dilute solutions (1:10) of this stock (but not stock solutions) lost enzyme activity rapidly when stored in polypropylene vials (but not glass vials), possibly owing to adsorption on the plastic (Randles & Armarego, 1985). Its activity was 81 nmol of NADH oxidized/min per 0.39 μ g in an assay mixture containing Tris/HCl buffer (100 μ l, 1 M), 6-methyl-5,6,7,8-tetrahydropterin (85 μ M), horseradish peroxidase (Boehringer grade II; 10 μ g), H_2O_2 (11 mM) and NADH (73 μ M) in a total volume of 1 ml at pH 7.4 (25 $^\circ C$) in the two cuvettes of a double-beam u.v. spectrometer, and the reaction was started by addition of 1 μ l of enzyme. The initial rate was measured by the change in absorbance at 340 nm by using ϵ_{340} 6.22×10^3 M $^{-1}$ ·cm $^{-1}$ for NADH.

Horseradish peroxidase

Boehringer grade II horseradish peroxidase (A_{403}/A_{279} ratio 1.0) or Sigma type I horseradish peroxidase (RZ 0.6, used at double the concentration) was used for the generation of quinonoid pterin species in the dihydropteridine reductase kinetic runs. Boehringer grade I horseradish peroxidase [A_{402}/A_{272} ratio 1.35, ϵ_{403} 63×10^3 M $^{-1}$ ·cm $^{-1}$ in 1 mM-phosphate buffer, pH 6.6, at 25 $^\circ C$; literature values (Schonbaum & Lo, 1972) A_{403}/A_{275} ratio 3.25, ϵ_{403} 102×10^3 M $^{-1}$ ·cm $^{-1}$] was used for the kinetic measurements, which showed that the tetrahydropteridines were viable substrates for peroxidase I (peroxidase- H_2O_2 complex).

Kinetic measurements

Solutions for the kinetic runs for obtaining the K_m and V_{max} values with peroxidase at 25 $^\circ C$ contained the following: Tris/HCl buffer, pH 7.4 (100 μ l, 1 M), [or potassium phosphate buffer, pH 7.1 (100 μ l, 3 M)], substrate (100 μ l, concn. 0.5–2 K_m), H_2O_2 (100 μ l, 11 or 22 nmol; stock solution concn. determined from $\epsilon_{237} = 67$ M $^{-1}$ ·cm $^{-1}$ in water) and water to make 990 μ l

(or 1 ml in the blank cuvette). The reaction was initiated by injection of peroxidase (10 μ l, 2 μ g of Boehringer grade I), and initial rates were obtained by measuring the rate of decrease in absorbance at an analytical wavelength set for each substrate (see Table 2). The non-enzymic rates in 0.1 M-Tris/HCl buffer, pH 7.4, were negligible for compounds (11), (12), (13) and (14) in the absence of H₂O₂, and 8.9, 7.0, 7.6 and 5.9% were oxidized respectively in the first 10 min in the presence of 11 mM-H₂O₂ alone. The amounts oxidized in the first 10 min were slightly less than these when H₂O₂ was 11 μ M.

Solutions for the kinetic runs for dihydropteridine reductase activity at 25 °C contained the following: Tris/HCl buffer, pH 7.4 (100 μ l, 1 M) [or potassium phosphate buffer, pH 7.1 (100 μ l, 3 M)], peroxidase (100 μ l, 20 μ g of Boehringer grade II or 40 μ g of Sigma type I enzyme), H₂O₂ (100 μ l, 11 μ mol), enzyme (1–3 μ l, 0.39–1.17 μ g, of stock or 10 times these values of 1:10-diluted enzyme, added to one cuvette), NADH (30 μ l, 2.7–4.4 mM; Sigma Chemical Co.; see Table 3), substrate (30 μ l, concn. 0.5–3 K_m) and water to make 1 ml. The first three components were made as a stock solution (kept at 0–2 °C during runs), enzyme was injected into one cuvette (with a Hamilton Whittier-cal PB600 dispenser with a 50 μ l Hamilton syringe delivering 1 μ l at a time), followed by simultaneous addition of NADH to both cuvettes (by placing the solutions in two small platinum baskets attached to a metal beam and moving them up and down in the solution eight times). After mixing, the recorder was activated to ensure that the absorbance remained unaltered for approx. 1 min. The reaction was initiated by simultaneous addition of substrate (as in the case of NADH addition above), and the initial rates were obtained from the rate of change of absorbance at 340 nm for NADH oxidation. Rates from duplicate runs from at least five concentrations of substrates (see above) were used to derive the kinetic parameters. All these parameters were calculated with the aid of a computer program kindly supplied by Dr. A. Cornish-Bowden (1981), and values derived from double-reciprocal plots of concentrations versus initial velocities agreed favourably with computed values. The pH values at the end of each run in this section were measured with a glass electrode and were identical with the values of the stock buffers originally used.

RESULTS AND DISCUSSION

Nature of the substrates for dihydropteridine reductase

The 5,6,7,8-tetrahydropteridin-4(3H)-ones [(11)–(13), p*K*_a approx. 3.8; cf. Albert & Matsuura (1962)] do not undergo aerobic oxidation in aqueous buffer at approx. pH 7 as readily as the more strongly basic 5,6,7,8-tetrahydropterins [e.g. (5) and (6), p*K*_a approx. 5.6; cf. Bobst & Viscontini (1966), Kaleen & Jencks (1968), Matsuura & Sugimoto (1970) and Pfeleiderer & Mengel (1971)] even though they exist entirely as neutral species. In this solution the pteridines (11)–(13) show virtually no changes in u.v. spectra in 1 h. Addition of 50–70 mol-equivalents of H₂O₂ causes changes in spectra, but they are too slow (less than 5% change in 10 min) for deriving kinetic parameters. However, when peroxidase and H₂O₂ are added together very rapid spectral changes occur (Fig. 1). The spectral changes for each compound are slightly different but all showed the same pattern, i.e. a fall in intensity of the major peak such that finally after 24 h the main u.v.-absorbing band had disappeared leaving a very weak band at long wavelength (approx. 380 nm). These are very similar to those observed with 4,5-diaminopyrimidin-6(1H)-one (14) and 2,4,5-triaminopyrimidin-6(1H)-one under the same conditions. The latter is known to give the quinonoid pyrimidine (15), which is a good substrate for dihydropteridine reductase (Armarego *et al.*, 1983b). These changes were found to obey first-order kinetics for more than 85% of the reaction, and the rate constants and half-lives are listed in Table 1.

Preliminary assays of the peroxidase/H₂O₂ oxidation products of compounds (11)–(14), without simultaneous addition of reagents to the reaction and reference cuvettes, indicated that the oxidation products were substrates for dihydropteridine reductase, but the reproducibility of the rate traces was very poor. We were

Fig. 1. U.v. spectra of 6-methyl-5,6,7,8-tetrahydropteridin-4(3H)-one (117 μ M) after treatment with peroxidase (20 μ g) and H₂O₂ (11 μ mol) in 0.1 M-Tris/HCl buffer, pH 7.4, at 25 °C

Scans 1–4 and 23 are after 0, 0.66, 2.3, 3.8 and 35 min respectively.

Table 1. First-order rate constants for the oxidation of compounds (11)–(14) with horseradish peroxidase/H₂O₂ at 25 °C

Reaction mixtures contained Tris/HCl (unless otherwise indicated (100 μ l; 1 M), substrate (100 μ l), H₂O₂ (100 μ l, 11 μ mol) and water (600 μ l), and the reaction was initiated with peroxidase (100 μ l; 20 μ g) at pH 7.3. Measurements were made with a single-beam spectrometer.

Compound	Final concn. (μ M)	Analytical wavelength (nm)	$10^{-3} \times k_{\text{obs.}} (\text{s}^{-1})$	$t_{\frac{1}{2}}$ (min)
(11)	149	289	4.95 (± 0.08)	2.3
(11a)	187	289	6.19 (± 0.07)	1.9
(12)	143	290	2.07 (± 0.02)	5.6
(12)*	117	290	1.92 (± 0.02)	6.0
(12)†	117	290	0.82 (± 0.01)	13.9
(12a)	173	292	2.10 (± 0.02)	5.5
(13)	172	292	2.12 (± 0.04)	5.5
(13)*	166	292	1.20 (± 0.01)	9.6
(13)†	166	292	0.39 (± 0.01)	29.4
(13a)	256	292	2.32 (± 0.04)	5.0
(14)	190	282	6.07 (± 0.01)	1.9
(14)‡	190	282	8.22 (± 0.01)	1.4
(14)†	190	282	7.11 (± 0.08)	1.6

* In 0.1 M-Bicine buffer, pH 7.0.

† In 0.3 M-potassium phosphate buffer, pH 7.0.

‡ In 0.1 M-Bicine buffer, pH 6.7.

concerned that this might be due to the rearrangement of the rapidly formed quinonoid species (8)–(10) to the assumed thermodynamically more stable 7,8-dihydropteridines (16, R¹ = H). In the pterin series this rearrangement occurs readily, e.g. (3) $\rightarrow$ (16, R¹ = NH₂, R² = R³ = Me, R⁴ = H), and is subject to general acid/base catalysis. It was also shown that when H-6 in alkylpterins [e.g. compounds (2) and (3)] was replaced by deuterium then the rate of rearrangement of quinonoid species to the 7,8-dihydro derivatives [e.g. (16), R¹ = NH₂] in Tris/HCl buffer at approx. pH 7 is subject to $k_{\text{H}}/k_{\text{D}}$ values as large as 10 (Archer & Scrimgeour, 1970; Armarego, 1984). We consequently synthesized the 6,7-dideuteriated derivatives (11a), (12a) and (13a) and examined the u.v. spectral changes immediately after oxidation with peroxidase/H₂O₂ as in the case of the respective protio compounds (11), (12) and (13). These also followed first-order kinetics for more than 85% of the reaction, and the rate constants are given in Table 1. Direct comparison revealed only small deuterium isotope effects, and the quinonoid dihydropteridines formed do not rearrange smoothly to the corresponding 7,8-dihydro derivatives as do the respective pterins. 7,8-Dihydropterins [i.e. (16), R¹ = NH₂] are subsequently oxidized to pterins, usually in good yields based on the original tetrahydropterin. With the pteridines (11)–(13), on the other hand, the yields of fully oxidized pteridin-4(3H)-ones isolated after peroxidase/H₂O₂ oxidations are exceedingly low (approx. 3–4%), and very little of the 7,8-dihydro derivatives is identified by t.l.c. Examination of the products of peroxidase/H₂O₂ oxidation of compounds (11)–(14) by removing samples at time intervals and separating the components by t.l.c. showed that within a minute of mixing at least seven spots appeared, and that their relative intensities varied with time but the number of spots did not decrease with time. The reaction was repeated on a larger scale for compounds (12) and (13), allowed to proceed for 24 h and the products were separated by t.l.c. One band in each case was identified

conclusively by u.v. spectra at three pH values, by ¹H-n.m.r. spectra and by t.l.c. (re-run) as the fully oxidized compounds (17) and (18). Most of the other bands gave substances, in small amounts, that had no u.v. absorption and a few unidentified proton signals in the n.m.r. spectra. Similar oxidations were performed in the n.m.r. tube and the proton spectra were examined at time intervals. After 3–5 min the downfield signal from H-2 disappeared whereas the upfield signals from the methyl groups persisted longer. These results indicate that oxidation at C-2 could have occurred to form, say, lumazines, and/or the pyrimidine ring was disrupted by hydration across the C-2=N-1 double bond followed by ring opening. The lack of u.v. absorption of some of the bands is indication of ring cleavage. In the case of the pyrimidine (14) similar results were obtained (i.e. several spots on t.l.c.), but we could not identify 2-carboxy-1,3,5-triazin-4(3H)-one or 1,3,5-triazin-2,4(1H,3H)-dione among the products. These would have been the possible products if the oxidation proceeded as that of 2,4,5-triaminopyrimidin-6(1H)-one (Jongejan *et al.*, 1983). We examined the oxidation with Br₂ and K₃Fe(CN)₆ with the aim of obtaining clear oxidation products of compounds (11)–(14), but in these cases also many products were formed, some of which had similar t.l.c. properties as those from the peroxidase/H₂O₂ oxidations. The results are consistent with the formation of a primary oxidation product (such as quinonoid dihydropteridine) that subsequently undergoes degradation to products that may react further with the oxidant.

Kinetic parameters for horseradish peroxidase

The structure of the quinonoid species (1)–(3) from the peroxidase/H₂O₂ oxidation of the tetrahydropterins (4)–(6), which are substrates for dihydropteridine reductase, is well established (cf. Armarego *et al.*, 1984). Although these species rearrange to the corresponding 7,8-dihydro(3H)pterins (16, R¹ = NH₂), they are stable

enough for some structural investigations. They can be generated so rapidly (e.g. within 20 s in assay conditions; see the Materials and methods section) that intermediates cannot be detected under the present assay procedures. The pteridin-4(3*H*)-ones (11)–(13) are also oxidized with peroxidase alone (or in buffer saturated with O₂), but very slowly (less than 0.1% in 10 min) compared with the oxidation of the pterins (5) and (6). The main difference in behaviour between the pterins and the pteridin-4(3*H*)-ones is that the quinonoid species from the latter are less stable and distinct species cannot be observed.

The close structural similarities of the tetrahydropterins (5) and (6) and the tetrahydropteridin-4(3*H*)-ones (12) and (13) prompted the suggestion that their oxidation products (2) and (3) and (9) and (10) should have the same structures and that differences in behaviour are due to the differences in oxidation potential between the two series (i.e. with and without the 2-amino group). In order to obtain further evidence to support this suggestion we tested both series of compounds to see if they were substrates for peroxidase/H₂O₂ and exhibited Michaelis–Menten kinetics. We found that this was the case. The u.v. spectrum of the peroxidase used was similar to that of the reduced enzyme, whereas in the presence of more than 1 equivalent of H₂O₂ it was converted into peroxidase I (cf. Schonbaum & Lo, 1972), a process that is known to be very fast (second-order rate constant $0.9 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$; Chance, 1949; Kato *et al.*, 1984). A preliminary study with a constant amount of peroxidase and H₂O₂ with various amounts of compounds (5), (6), (11), (12), (13) and (14) revealed that the rates of oxidation of all these compounds exhibited saturation kinetics, i.e. the rates increased with increasing concentration of substrate until they reached a maximum value. A detailed investigation was undertaken of the rates of oxidation of all these substrates with various amounts of peroxidase and of H₂O₂, and keeping the concentration of one of these two components constant while altering that of the other. A typical series of curves is shown in Fig. 2. The rates increase linearly up to a concentration of peroxidase of 8 $\mu\text{g}/\text{ml}$. The rates increased as the H₂O₂ concentration increased up to approx. 100 μM , and then the rates decreased, most probably owing to deterioration of the

enzyme (Schonbaum & Lo, 1972). For the measurements of the kinetic parameters we settled on a low concentration of H₂O₂ (11 μM). From the plots of this concentration of H₂O₂ we chose enzyme concentrations from the linear portions of the curve where the slope was such that doubling the amount of enzyme doubled the rate of reaction. The kinetic parameters for the enzyme with the above substrates are listed in Table 2, together with the concentrations of the reagents used.

The K_m and k_{cat} values obtained show that the enzymic oxidation of all these substrates obeys Michaelis–Menten kinetics. The orders of these parameters imply

Fig. 2. Effect of peroxidase (Sigma type I) and H₂O₂ concentrations on the initial rates of oxidation of 6,7-dimethyl-5,6,7,8-tetrahydropteridin-4(3*H*)-one at the various concentrations shown in 0.1 M-Tris/HCl buffer, pH 7.4, at 25 °C

Table 2. Kinetic parameters of horseradish peroxidase/H₂O₂ with the pteridines (11)–(13), pyrimidine (14) and pterins (5) and (6) at pH 7.4 and 25 °C

Reaction mixtures contained Tris/HCl (unless otherwise indicated) (100 μl ; 1 M, substrate (100 μl , in 4 mM-HCl), H₂O₂ (100 μl ; 110 μM) and water to make a final volume of 1.000 ml, and the reaction was initiated by adding peroxidase (Boehringer grade I) (2 μg in 10 μl to one cuvette or 0.2 μg in 10 μl with the pterins). For k_{cat} , the effective concentration of enzyme was calculated to be as 61.8% from $\epsilon_{403} = 63 \times 10^{-3} \text{ M}^{-1} \cdot \text{cm}^{-1}$ [literature value (Schonbaum & Lo, 1972) $\epsilon_{403} = 102 \times 10^{-3} \text{ M}^{-1} \cdot \text{cm}^{-1}$].

5,6,7,8-Tetrahydropteridin-4(3 <i>H</i>)-one	K_m (μM)	V_{max} ($\mu\text{mol}/\text{min}$ per mg)	k_{cat} (s^{-1})	Analytical wavelength (nm)
Unsubstituted (11)	173 (± 3)	143 (± 1)	58.6 (± 0.5)	289
6-Methyl- (12)	314 (± 20)	53.4 (± 2)	22.0 (± 0.9)	290
<i>cis</i> -6,7-Dimethyl- (13)	44.9 (± 0.2)	16.1 (± 0.03)	6.6 (± 0.01)	292
	28.6 (± 0.4)*	11.6 (± 0.09)*	4.8 (± 0.04)*	292
4,5-Diaminopyrimidin-6(1 <i>H</i>)-one (14)	203 (± 8)	149 (± 3)	55.6 (± 1.4)	282
5,6,7,8-Tetrahydropterin				
6-Methyl- (5)	45.7 (± 1.1)	27.9 (± 0.4)	11.5 (± 0.1)	298
<i>cis</i> -6,7-Dimethyl- (6)	103 (± 2)	50.9 (± 0.8)	21.0 (± 0.3)	298

* In 0.3 M-potassium phosphate buffer, pH 7.1.

that the mechanisms of enzymic oxidation of the tetrahydropteridin-4(3*H*)-ones (11), (12) and (13) are probably similar to those of the tetrahydropterins (5) and (6), and that the initial products formed are the quinonoid species (8)–(10), (2) and (3) respectively.

Kinetic parameters for dihydropteridine reductase

In the first enzymic experiments we ventured to see if the peroxidase/H₂O₂ oxidation products of compounds (11)–(13) were inhibitors of dihydropteridine reductase. We found no inhibition at concentrations of 120–140 μ M of the products when quinonoid 6-methyl-7,8-dihydro-(6*H*)pterin (2) (at 49 μ M) and NADH (at 58 μ M) were used. Incubation of compounds (11)–(13) in the presence of peroxidase/H₂O₂ for various times up to 6 half-lives (see Table 1) also showed no decrease in the initial rates with quinonoid pterin (2) and NADH. Preliminary studies, however, showed that one of the products of the peroxidase/H₂O₂ oxidation products of each of compounds (11)–(14) was a substrate for dihydropteridine reductase.

The kinetic properties of these substrates (shown in Table 1) made it difficult to use standard mixing procedures because the decomposition of the initial quinonoid species formed can proceed to quite an extent in one cuvette before addition of an ingredient in the other cuvette. It was imperative that addition of both the substrates (11)–(14) and the NADH solutions were respectively added simultaneously to both cuvettes. After experimenting also with the order of addition of reagents we selected the order that gave best reproducibility of initial rate traces for a particular series of runs. Because the initial products of peroxidase/H₂O₂ oxidation of the substrates (11)–(14) undergo decomposition readily, it was necessary to find out how the dihydropteridine reductase activity altered with time after allowing the decomposition to proceed before starting the assay for this enzyme. In this case the order of addition was stock solution (Tris/HCl, peroxidase, H₂O₂), dihydropteridine reductase (in one cuvette only), substrate (11)–(14) (simultaneously to both cuvettes) and allowing a recorded time interval before initiation of the reductase

reaction by adding NADH (simultaneously to both cuvettes) and recording the spectral change at 340 nm. The curve for 6-methyl-5,6,7,8-tetrahydropteridin-4(3*H*)-one is shown in Fig. 3. Maximum activity was also observed at the shortest time with compounds (11), (13) and (14), and all subsequent assays were performed at the shortest possible time (approx. 1 min). The same procedure was used for the measurement of the kinetic parameters except that the order of simultaneous addition of compounds (11)–(14) and NADH was reversed, i.e. the reactions were initiated with compounds (11)–(14). In this order, the oxidation of the tetrahydropteridines by peroxidase/H₂O₂ may appear to be inhibited by large concentrations of NADH. This is because the quinonoid dihydropteridine is reduced back

Fig. 3. Dihydropteridine reductase (59 ng) activity at various time intervals after initiation of the peroxidase/H₂O₂ oxidation of 6-methyl-5,6,7,8-tetrahydropteridin-4(3*H*)-one (988 μ M) and starting the reaction with NADH (79 μ M) in 0.1 M-Tris/HCl buffer, pH 7.4 (total volume 1 ml), at 25 °C

Table 3. Kinetic parameters for compounds (8)–(10) with human brain dihydropteridine reductase at 25 °C

Solutions were mixed in the order buffer (100 μ l; 10 times the concentration given below), peroxidase (100 μ l; 40 μ g), H₂O₂ (100 μ l; 11 μ mol) and water to make 1.000 ml after all ingredients are added (these four are used as a stock solution), dihydropteridine reductase (7 μ l of 1/10-diluted enzyme, to 3 μ l of stock enzyme injected, to one cuvette only), NADH (30 μ l, simultaneously to the two cuvettes) and allowed to equilibrate. Reaction is initiated by addition of tetrahydropteridines (11)–(13) (30 μ l) simultaneously to both cuvettes.

Substrate	Apparent K_m (μ M)	Apparent V_{max} (μ mol/min per mg)	Concn. of NADH (μ M)	Concn. of enzyme (μ g/ml)
0.1 M-Tris/HCl buffer, pH 7.4				
(8) from (11)	669 ($\pm$ 2)	234 ($\pm$ 0.4)	117	0.39
(9) from (12)	366 ($\pm$ 20)	131 ($\pm$ 3)	117	0.39
(10) from (13)	754 ($\pm$ 45)	76 ($\pm$ 4)	117	1.17*
0.3 M-Potassium phosphate buffer, pH 7.1				
(8) from (11)	597 ($\pm$ 20)	318 ($\pm$ 8)	117	0.39
(9) from (12)	276 ($\pm$ 8)	444 ($\pm$ 7)	80	0.27
(10) from (13)	640 ($\pm$ 52)	489 ($\pm$ 27)	131	0.31

* Close to, but not on, the linear part of the plot of rate versus enzyme concentration.

to the starting tetrahydropteridines by NADH, the reduction being faster the higher the concentration of NADH and pteridine. We have studied the rates of non-enzymic reduction of the quinonoid species (2), (3) and (8)–(10) with the use of a single-beam spectrometer and found that at pH 7.4 the rates were 1.4–6.8% of the enzymic rates. The rates of formation of quinonoid species are not rate-limiting. Note also that the non-enzymic rates are compensated in the double-beam spectrometer and are neglected, and hence only the enzymic rates are observed. Below pH 6.8, however, the non-enzymic rates increase sharply because the quinonoid species become protonated (pK_a approx. 5.4 for the pterins; see below). These compounds were clearly oxidized to transient species that exhibited Michaelis–Menten kinetics with dihydropteridine reductase and the appropriate apparent K_m and V_{max} values that we measured are in Table 3.

For meaningful kinetic parameters the concentrations of dihydropteridine reductase were selected from plots of enzyme activity versus enzyme concentration. In all cases the plots were linear up to enzyme concentrations of 0.4–0.6 mg/ml, i.e. the rate was doubled when the enzyme concentration was doubled, and then slowly levelled off. This levelling off of enzyme activity does not appear to be caused by inefficient recycling of the pteridines by peroxidase/ H_2O_2 and may be due to lower effective concentration of enzyme caused by association or polymerization, but we have not investigated this further. The enzyme concentrations used were selected from the linear portions of the plots. Although we found that the quinonoid species of the 4,5-diaminopyrimidinone (14) had substrate activity, these decomposed too rapidly (see Table 1), and the reproducibility of the initial-rate plots was very poor.

The parameters were measured in Tris and phosphate buffers because it was found that the rates of oxidation were lower in the latter buffer. We do not know precisely what is happening in this reaction, but we know that the quinonoid species are formed rapidly and then decompose. The plot in Fig. 3 confirms this because the dihydropteridine reductase activity of the species formed from peroxidase/ H_2O_2 oxidation is highest immediately (approx. 1 min) after mixing and then decreases. The rate constants in Table 1 are therefore for the formation and degradation of the quinonoid species. Since we do not know the individual rate constants of formation and degradation of the active species, we cannot take the concentrations of the respective quinonoid species in Table 3 as being the same as those of the tetrahydropteridines (11), (12) and (13) from which they were derived. Therefore the apparent K_m values are maximum values for these substrates and the true values could be much smaller. The reproducibility of the initial rates and the satisfactory spread of errors confirms that the proportional concentrations of quinonoid species with respect to tetrahydropteridines used, at various concentrations of the latter, are within experimental error in each set of experiments.

The K_m values in phosphate buffer are slightly less than in Tris buffer and could reflect the higher proportion of quinonoid species (slower decomposition) in the former buffer. This does not affect the apparent V_{max} values, which are higher in phosphate buffer. The concentrations of NADH in Table 3 are at or close to saturating values because when they are doubled no significant increases in

rates are observed. Some of the substrates contained small amounts of borate salts, and we were concerned that borate might inhibit the enzyme. No inhibition of dihydropteridine reductase was observed at borate concentrations up to 1 mM when 6-methyl-7,8-dihydro(6*H*)-pterin (2) (at 55 μM) and NADH (100 μM) were used. The highest concentration of borate from the substrates in the assays was 0.9 mM, and most of the solutions contained considerably less than this concentration.

In conclusion, we have shown that the 5,6,7,8-tetrahydropteridin-4(3*H*)-ones (11), (12) and (13) are effective substrates for horseradish peroxidase in the presence of H_2O_2 , similarly to the related 2-amino derivatives, the 5,6,7,8-tetrahydropterins (5) and (6). The transient quinonoid species derived from the oxidation of the tetrahydropteridin-4(3*H*)-ones (11)–(13) are found to be substrates for dihydropteridine reductase. A 2-amino group is not essential for substrate activity with the latter enzyme but most probably enhances the binding. The data show that the quinonoid structures (2) and (3), but not (7), are probably the reactive species in the dihydropteridine reductase-catalysed oxidation of NADH. The proposition that the quinonoid structures derived from compounds (11)–(14) are different from those derived from the pterins (2) and (3) when the same oxidant (peroxidase/ H_2O_2) is used, and are also viable substrates for a second enzyme (dihydropteridine reductase), is very unlikely. Such compounds, however, could have the *ortho*-quinonoid structure (19), a structure with energy comparable with that of the tautomers (2) and (3) (Gready, 1985), but this has been ruled out by other studies (Armarego & Waring, 1982; Lazarus *et al.*, 1982; Benkovic *et al.*, 1985). The u.v.-spectral changes of 2,5-diaminopyrimidin-4(3*H*)-one (20) on treatment with peroxidase/ H_2O_2 are very similar to those of the pyrimidinone (14) and the pteridines (11)–(13) (see Fig. 1), and would exclude a quinonoid species with bond arrangements such as (19). Moreover, the pyrimidinone (20) after peroxidase/ H_2O_2 treatment exhibited some activity with dihydropteridine reductase. Structure (19) for the pteridin-4(3*H*)-ones implies a remote possibility that dihydropteridine reductase can reduce two different tautomers of very similar substrates. It is true that the cations of the two tautomers are mesomeric, (21) $\rightleftharpoons$ (22), and protonation of the quinonoid species will enhance hydride transfer to N-5 considerably. However, the enzyme will still need to transfer a proton to N-3 in one case (the pterins) and to N-8 in the other case (the pteridinones) after binding of the neutral species, if one postulates different tautomeric structures. If the pteridine binds as the cation, on the other hand, it does not matter which tautomer predominates in neutral solution. The pK_a of the quinonoid dihydropterin (3) is 5.4 (W. L. F. Armarego, unpublished work; cf. also Bailey & Ayling, 1983) and at pH 7.4 it is almost entirely in the unprotonated form. The absence of a 2-amino group from the corresponding quinonoid dihydro(6*H*)pteridin-4-one (10) would be base-weakening and make its pK_a lower than 5.4 by at least 1.6 pH units [compare the pK_a values of 5,6,7,8-tetrahydropteridin-4(3*H*)-ones approx. 3.8; Albert & Matsuura, 1962) and 5,6,7,8-tetrahydropterins (approx. 5.6; Matsuura & Sugimoto, 1970; Pfeleiderer & Mengel, 1971)]. These species are even less likely to be protonated at pH approx. 7.4. Protonation of quinonoid species would most probably take place on the enzyme after or before hydride transfer, but this informa-

tion is not known at present. Pulse-chase and stopped-flow kinetic studies of the mechanism of the reactions at the active site of dihydropteridine reductase have revealed that NADH binds first and very tightly, followed by the quinonoid dihydropterin, and that the rate-determining step is a conformational change of the ternary complex, probably assisting in or associated with substrate protonation, immediately before the faster hydride transfer (no deuterium isotope effect) from NADH to the quinonoid dihydropterin, followed by release of products (Poddar & Henkin, 1984). A study of the profiles of the kinetic parameters plotted versus pH could tell us more about the details of the protonation process. Finally, we have examined 6-methyl-7,8-dihydro(6H)lumazine (23), which is relatively stable and whose structure has been established by n.m.r. spectroscopy (Lazarus *et al.*, 1982), and found that at 41 μM it is neither a substrate nor an inhibitor of this enzyme.

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