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Abbreviations

H_4DMP 6,7-dimethyltetrahydropterin

DCIP dichloroindophenol

MV^+ methyl viologen radical cation

Abstract

This work provides the first extensive study of the redox reactivity of the pyranopterin system that is a component of the catalytic site of all molybdenum and tungsten enzymes possessing

molybdopterin. The pyranopterin system possesses certain characteristics typical of tetrahydropterins, such as a reduced pyrazine ring, however it behaves as a dihydropterin in redox reactions with oxidants. Titrations using ferricyanide and DCIP (dichlorophenylindophenol) prove a 2 e^- / 2 H^+ stoichiometry for pyranopterin oxidations. Oxidations of pyranopterin by $Fe(CN)_6^{3-}$ or DCIP are slower than tetrahydropterin oxidation under a variety of conditions but are considerably faster than observed for oxidations of dihydropterin. The rate of pyranopterin oxidation by DCIP was studied in a variety of media. In aqueous buffered solution, the pyranopterin oxidation rate has minimal pH dependence whereas the rate of tetrahydropterin oxidation decreases 100-fold over the pH range 7.4 - 8.5. Although pyranopterin reacts as a dihydropterin with oxidants, it resists further reduction to a tetrahydropterin. No reduction was achieved by catalytic hydrogenation, even after several days. The reducing ability of the commonly used biological reductants dithionite and methyl viologen radical cation was investigated but experiments show no evidence of pyranopterin reduction by any of these reducing agents. This study illustrates the dual personalities of pyranopterin and underscores the unique place that the pyranopterin system holds in the spectrum of pterin redox reactions. The work presented here has important implications for understanding the biosynthesis and reaction chemistry of the pyranopterin cofactor in molybdenum and tungsten enzymes.

Keywords

Molybdopterin

Pyranopterin

Molybdenum enzymes

Molybdenum cofactor

Introduction

The tricyclic pyranopterin structure is a component of the catalytic site of all molybdenum and tungsten enzymes except nitrogenase[1-4]. Specifically, the ligand bound to Mo and W in these enzymes is a dithiolene chelate extending from the pyran ring of a pyranopterin (Fig. 1). Together the metal and ligand ensemble is called the molybdenum (or tungsten) cofactor and these cofactors within molybdenum and tungsten enzymes are found in all organisms from archeabacteria to humans. The dithiolene-pyranopterin ligand has acquired its own name, *molybdopterin*, whose prefix "molybdo" was chosen to denote the special pterin ligand for Mo in molybdoenzymes.

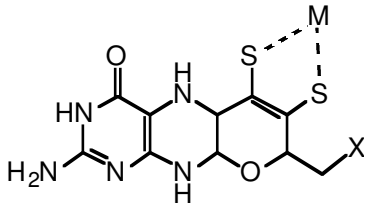

Fig. 1. A minimal depiction of the molybdopterin ligand that binds Mo or W in molybdenum and tungsten enzymes. X represents the variable group which is either a phosphate or a dinucleotide.

Evolutionary conservation of the molybdopterin ligand is strong evidence that it serves an important function to the metal site in catalysis. Features unique to a dithiolene chelate which might be important to the Mo and W cofactors have been identified by a considerable number of coordination chemistry studies comparing dithiolene with other sulfur donor ligands [5-12].

Less well understood are those features of the pyranopterin which could explain its presence in the cofactors.

Two roles for the pyranopterin within molybdopterin are evident from protein X-ray structures [4]. The wealth of amide, amino and imine functional groups that characterize the pterin ring system serve to anchor the cofactor by extensive hydrogen bonding to the protein. Crystallography has also revealed that in many cases the pyranopterin is H-bonded to other electron transfer groups, particularly Fe_2S_2 and Fe_4S_4 clusters [4,13,14]. Thus it appears that molybdopterin fills both a structural as well as an electron transfer role.

Pterins have key roles elsewhere in biochemistry in addition to the ubiquitous pyranopterin of the Mo and W enzymes. In most of these cases, the pterin group participates in redox reactions where the tetrahydro/dihydro redox reaction is coupled to substrate catalysis [15-17]. Accordingly, the discovery of the pterin component in the Mo cofactor and its initial identification as a fully reduced tetrahydropterin suggested a likely role in electron transfer [18, 19]. Subsequent X-ray crystal structures from different enzymes in various forms have all shown the molybdopterin ligand to possess not a tetrahydropterin but a tricyclic pyranopterin structure [4,13].

Even though both pyranopterin and a tetrahydropterin display similar tetrahydropyrazine rings, (compare 6,7-dimethyltetrahydropterin **A** and the pyranopterin **D** in Fig. 2) the possibility of pyran ring opening alters the potential products from pyranopterin redox reactions. The ring-opened pyranopterin is at the dihyro-level of reduction, a reduction state two hydrogen atoms oxidized compared to tetrahydropterin . Although this important difference between

pyranopterin and tetrahydropterin has been addressed [20], there is scant experimental data available on redox transformations of pyranopterin [21].

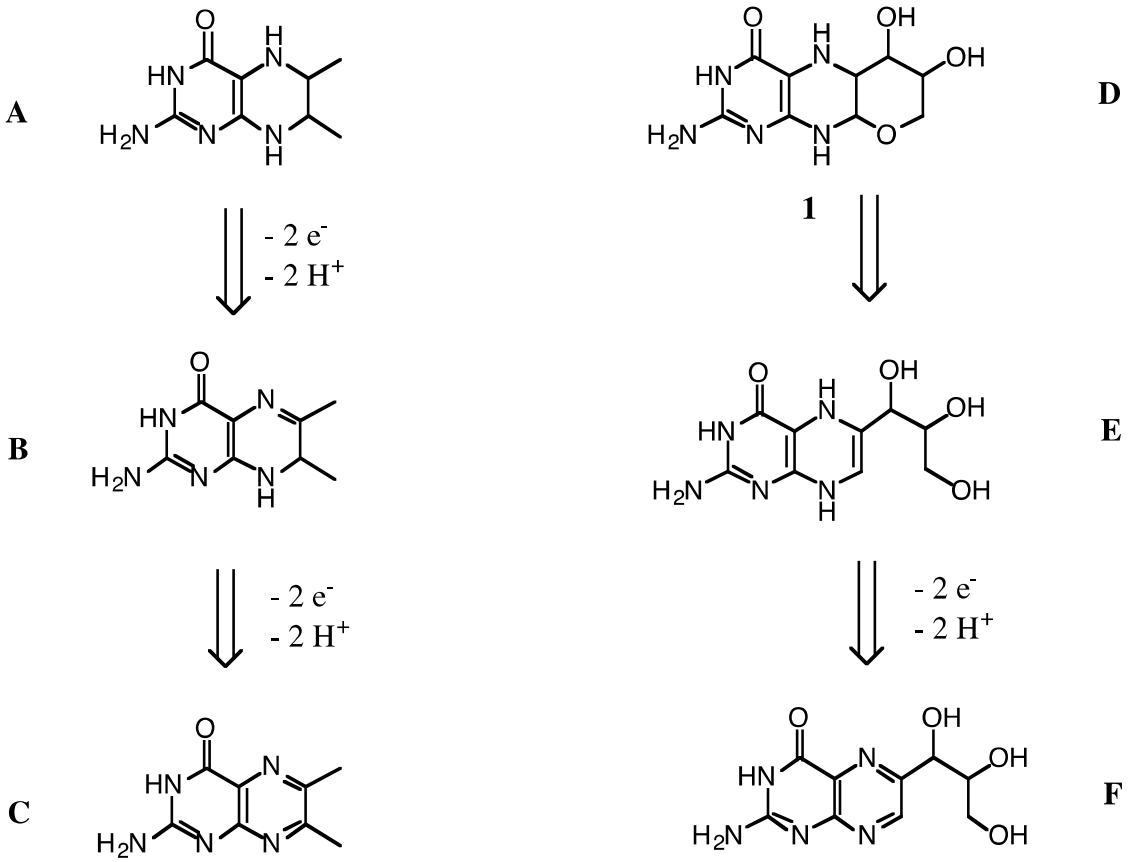

Fig. 2. A comparison of the oxidation reactions of 6,7-dimethyltetrahydropterin **A** and 1',2'-dihydroxypyranopterin **D**. The species illustrated are: **B**, 6,7-dimethyl-7, 8-dihydrotetrahydropterin; **C**, 6,7-dimethylpterin; **D,** pyranopterin; **E,** 5,8-dihydroneopterin, one of several possible tautomers; **F,** neopterin.

The focus of this study is to explore the redox chemistry of the pyranopterin system. The context that frames our study is the known oxidation and reduction reactions within the simpler pterin

system [22]. Our goals are to investigate oxidation and reduction reactions of pyranopterin, identify the reaction products, determine the reaction stoichiometry, and to compare the reactivities of reduced species in the bicyclic pterin and tricyclic pyranopterin systems. Our results show that 1',2'-dihydroxypyranopterin **1** undergoes a two-electron, two-proton oxidation to neopterin, a fully oxidized pterin. The rate of pyranopterin oxidation is slower than tetrahydropterin under a variety of conditions. Most importantly, pyranopterin oxidation *irreversibly* destroys the pyran ring and converts it into the bicyclic pterin system. This latter observation has critical significance with respect to the requirement to protect molybdopterin and its pyranopterin structure from oxidation during both biosynthesis [23] and cofactor isolation [24].

Materials and Methods.

All chemical reagents were purchased from Sigma-Aldrich Chemical Co. Solvents of HPLC grade were deaerated with N_2 and stored over 4Å molecular sieves except for methanol that was stored over 3Å molecular sieves. Anhydrous diethylether was purchased and not further purified. NMR spectra were recorded on an IBM 300 MHz FT-NMR and referenced to the chemical shifts of deuterated solven. A Hewlett-Packard 8452A spectrophotometer was used to obtain electronic absorption spectra.

Synthesis of Pyranopterin 1. L-Arabinose-phenylhydrazone [25-27] (1.16 mmol, 2.79 g) and 2,5,6-triaminopyrimidine-4(3H)-one dihydrochloride (0.934 mmol, 2.00 g) were placed into a Schlenk flask under nitrogen. Methanol (130 mL) and 2-mercaptoethanol (0.250 mL) were transferred to the reaction flask and stirred for 1.5 h at ambient temperature. The reaction

mixture was then refluxed under N_2 for 2 h and cooled at 4°C overnight. A Schlenk funnel with medium frit was filled with 150 mL of nitrogen-purged diethylether and the reaction mixture was transferred directly into this ether via cannula. A fine hygroscopic orange powder was collected and washed with diethylether until the filtrate was colorless. The powder was decolorized with activated charcoal in warm 2N HCl/MeOH (45°C) following by precipitated with Et_2O. This yellow precipitate was transferred to a glove box where it was recrystallized twice with methanol/ethyl ether until a pale yellow solid was obtained.

Catalytic hydrogenation was used to remove the trace amounts of oxidized pterin from the product as follows: 0.50 g of the yellow solid was dissolved in 10.0 mL of MeOH and 50.0 mL of 2.0 N HCl and 0.20 g of 5%Pd/C catalyst was added. The reaction mixture was placed under 40 psi of H_2 and hydrogenated in a Parr hydrogenator overnight. The catalyst was filtered off and the solution was reduced by rotary evaporation. A dark yellow oil was transferred into the dry box. The oil was dissolved in MeOH and a white-yellow solid precipitated out with diethyl ether. Elemental analysis: $C_9H_{15}N_5O_4$•3HCl: Theory; %C: 32.94, %H: 4.61, %N: 21.34; Actual; %C: 33.30, %H: 4.47, %N: 21.70.

Oxidation of Pyranopterin to Neopterin. Crude pyranopterin (0.28 g, 1.1 mmol) was dissolved in 35 mL MeOH. Aerobic oxidation was accelerated with approximately 3 mL of a 5% H_2O_2 solution. The pyranopterin oxidation was monitored by UV spectroscopy where absorption changes at 334, 348 and 364 nm indicated the formation of oxidized pterin. Addition of diethyl ether (14 mL) caused precipitation of neopterin, which was collected by vacuum filtration and washed with methanol and ether. Yield: 35%.

Typical Oxidation Kinetics Protocol. All preparations and manipulations of reaction solutions were performed under nitrogen in a glove box. Pterin (**1** or H_4DMP) and dichloroindophenol (DCIP) were dissolved separately in TRIS buffer at concentrations of 0.50 mM. For a typical kinetic run, 0.75 mL of the above pterin solution and 1.5 mL buffer were loaded into a cuvette capped by a septum. Immediately prior collecting absorbance data, 0.75 mL of the DCIP solution was added via syringe. The cuvette was inverted to mix the solutions and absorbance measurements were made every 3 sec for 150 sec. The value of A**.** was measured 10 min after DCIP injection. Plots of $1/(A_t - A_{\infty})$ were linear over the first half-life and used to extract an observed second order rate constant, k_{obs}, $mol^{-1}L\ sec^{-1}$. The extinction coefficient of DCIP incorporated into the k_{obs} varies with pH and was determined by Beer's law plots: ε, $mol^{-1}L\ cm^{-1}$: 13,000 (pH 7.40); 14,800 (pH 7.68), 14,400 (pH 8.04), 9,100 (pH 8.45).

The above protocol was also followed for kinetic experiments in other media. The extinction coefficient of DCIP in organic solvents was determined by Beer's law plots. ε_{λ}, $mol^{-1}L\ cm^{-1}$ (solvent): ε_{644} 7840 (DMF); ε_{630} 23,400 (MeOH).

Attempted Catalytic Reduction of Pyranopterin to Tetrahydroneopterin. In a typical procedure, **1** (0.23 g, 0.707 mmol.) was dissolved in 50.0 mL of aqueous 2N HCl, degassed, and bubbled with N_2, then 0.100 g of 5% Pd on activated carbon catalyst was added to the solution. The reaction mixture was placed under 40 psi of H_2 and shaken for 36 hours in a Parr hydrogenator system. After removing the catalyst by filtration, the solution volume was reduced by rotary evaporation to produce a dark yellow oil. This oil was transferred into the dry box and dissolved in a minimal amount of MeOH. Addition of diethylether caused precipitation of a

white-yellow solid (0.21 g, 90% recovery). The product was recrystallization from a mixture of cyclohexane:toluene (50:50 v/v.) and washed with diethylether. Analysis of the product by ^{1}H NMR confirmed no reduction of pyranopterin.

Variations in this procedure include: 1) increasing the hydrogenation period to 7 days, 2) use of a MeOH/2 N HCl solvent mixture (1:5 v/v) in a 24 h reaction, 3) increasing the amount of Pd/C catalyst (0.200 g). Analysis of the product by ^{1}H NMR showed no reduction of pyranopterin under any of these conditions.

Typical Reduction Titration Procedure. Solutions of pyranopterin, methyl viologen dichloride and sodium dithionite were prepared in distilled, deionized water or in TRIS buffer at pH 7.0. Under a nitrogen atmosphere, aliquots of a stock solution of **1** (0.15 mM) were added to cuvettes containing 0.1 mM methyl viologen and 0.05 mM dithionite. The absorbance at 396 and 600 nm were measured and plotted as a function of [oxidant] (where oxidant is either **1** or DCIP). This titration procedure was repeated using DCIP a oxidant as a control experiment to confirm the 2:1 MV^{+} :DCIP reaction stoichiometry.

Results

Syntheses. Pyranopterin **1** was obtained by an anaerobic modification of the method of Pfleiderer who first reported its synthesis albeit as an unstable molecule [28, 29]. The synthesis of **1** proceeds through condensation of L-arabinose-phenylhydrazone and triaminopyrimidone followed by intramolecular cyclization. A purification method was developed using catalytic

hydrogenation followed by recrystallization to remove the contaminant neopterin formed by adventitious oxidation of **1**. Pfleiderer's prior work established that pyranopterin **1** is in equilibrium with a furano isomer (Fig.3) where NMR studies showed the equilibrium lies in favor of the pyrano- isomer in a 3:1 (pyrano:furano) ratio [28]. Although the pyrano form can be isolated by a process of acetyation, HPLC purification and deacetylation [28,29], once the pyranopterin is placed into solution the furano/pyrano equilibrium is rapidly reestablished. Acetylated **1** has been structurally characterized by X-ray diffraction [5]. The synthesis yields two diastereomers of **1**, (R, S) and (S, R) which can be separated after conversion to tetraacetylated derivatives. For the purposes of this study these diastereomers were not separated.

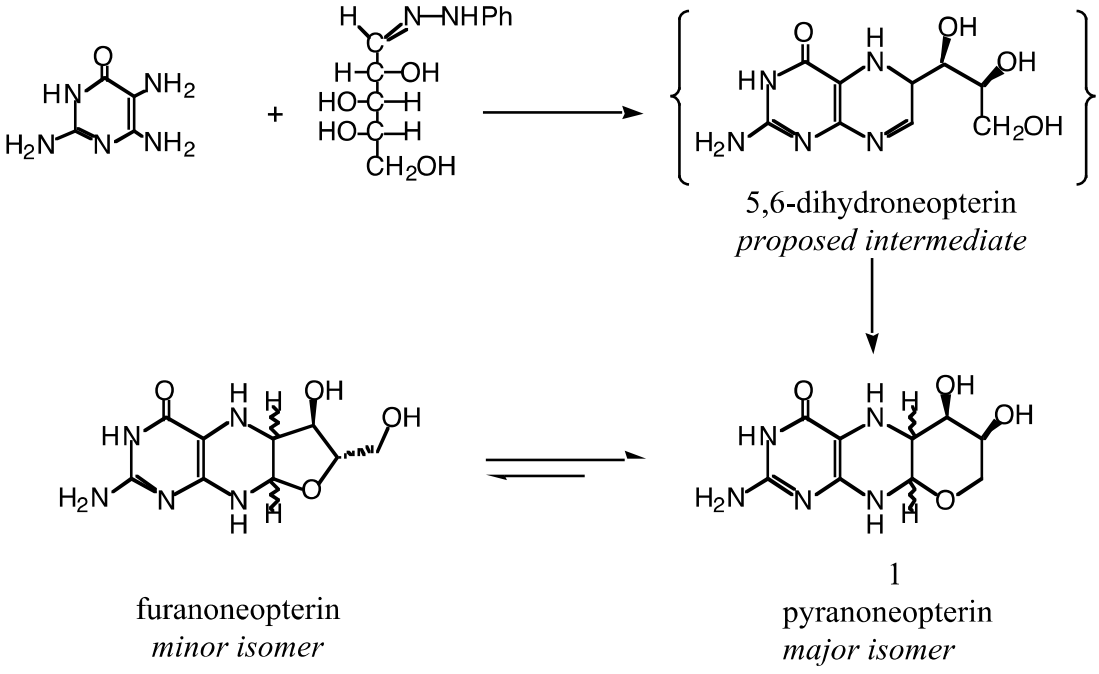

Fig. 3. Synthetic route to pyranopterin 1 used in this study as reported by Pfleiderer [28,29].

Neopterin was synthesized and used in this study as a control to verify the product of pyranopterin oxidation. Neopterin is formed directly from air oxidation of pyranopterin, however, we found that it is more easily isolated from hydrogen peroxide oxidation of pyranopterin.

Oxidation Reactions. The oxidation of **1** by ferricyanide and by the redox dye dichloroindophenol (DCIP) was investigated since these oxidants were previously used to probe the redox level of molybdopterin in the molybdoenzymes sulfite oxidase [30, 31]. Oxidized $DCIP_{ox}$ is blue (630 nm) and can be reduced by two electrons to the colorless reduced $DCIP_{red}$. A titration of **1** with $DCIP_{ox}$ can be easily monitored by electronic spectroscopy at 630 nm. The titration plot in Fig. 4 shows that at the DCIP:pyranopterin mole ratio of 1:1, A_{630} begins to increase and signals the endpoint of the redox reaction. The titration plot shows that the reaction stoichiometry is 1:1. Since $DCIP_{ox}$ is a two-electron oxidant, the reaction stoichiometry indicates that pyranopterin has been oxidized by two hydrogen atoms to neopterin. The product neopterin was identified by ^{1}H NMR by comparison with an authentic sample. Similarly, two equivalents of the one-electron oxidant ferricyanide are required to produce neopterin from pyranopterin (identified by ^{1}H NMR). These results prove that, despite the structural similarity to a tetrahydropterin, the observed reactivity of pyranopterin is that of a dihydropterin, not a tetrahydropterin, in accordance with Fig. 2.

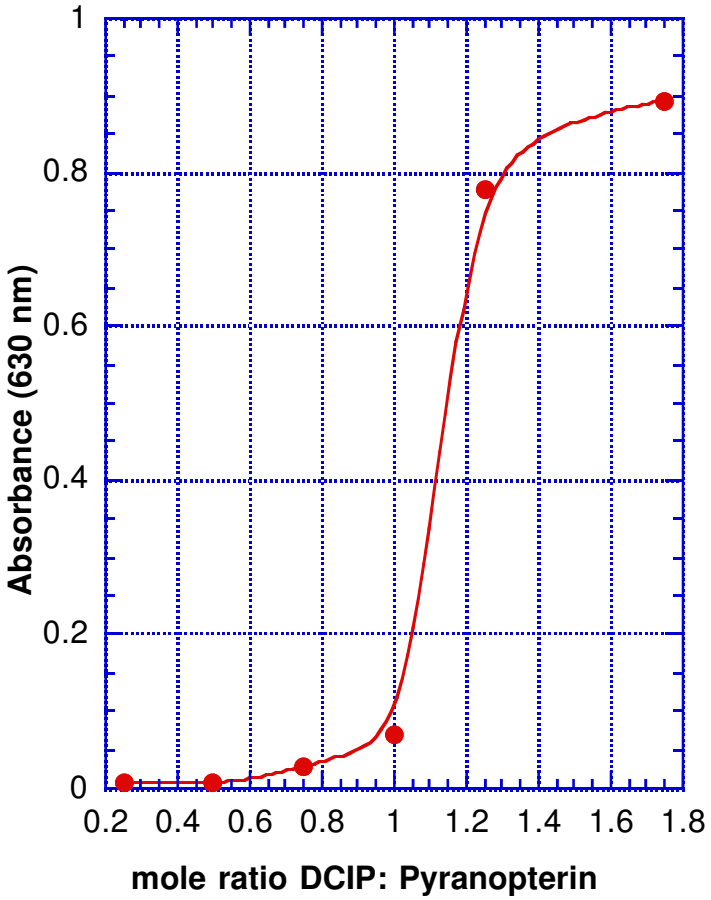

Fig. 4. A titration plot for the oxidation of **1** by $DCIP_{ox}$ monitoring the appearance of excess $DCIP_{ox}$ at 630 nm.

The relative ease of pyranopterin oxidation by DCIP was compared with that of 6,7-dimethyltetrahydropterin H_4DMP in kinetics experiments. Second order plots of $1/(A_t - A_\infty)$ vs time were linear over the first half-life (data not shown) and used to extract an observed second order rate constant k_{obs} (Table 1).

Values of k_{obs} reveal the effect of solvent environment on pterin oxidation rate. The solvent environment was varied in these investigations and included the organic solvents methanol and

dimethylformamide and aqueous TRIS buffered solutions in the pH range 7.40 - 8.45. Fig. 5 graphically illustrates that the rate of pyrano- and tetrahydro-pterin oxidation is strongly dependent on the solvent medium and spans four orders of magnitude.

Table 1. Second Order Rate Constants, k_{obs} (mol^{-1} L sec^{-1}), for DCIP oxidation of H_4DMP and Pyranopterin **1** in TRIS buffer and in several organic solvents at 25 ^{o} C.

	H_4dmp	**1**
pH[1]	k_{obs} x 10^{-3}	k_{obs} x 10^{-2}
7.40	226	5.1
7.68	14.7	3.6
8.04	9.3	5.7
8.45	3.8	5.3
[a]DMF	0.023	0.10
[b]DMF	5.2	4.5
MeOH	0.38	0.82

[a]DMF is purified by distillation.

[b] DMF is HPLC grade solvent stored over 4 Å molecular sieves.

Fig. 5 shows that, in every case, tetrahydropterin is more rapidly oxidized by DCIP than pyranopterin but the magnitude of the difference is strongly solvent dependent. Thus, H_4DMP oxidation is twice as fast as pyranopterin oxidation in distilled dimethylformamide but forty times faster in aqueous solution at pH 7.68 (Table 1). Fig. 6 shows the rate comparison of tetrahydopterin and pyranopterin oxidations in TRIS buffered aqueous solutions when pH varies from 7.4 to 8.5. In this near-neutral pH range, pyranopterin exhibits little change in oxidation

rate. In contrast, the oxidation of tetrahydropterin H_4DMP is strongly accelerated by 60-fold at the low end of this pH range.

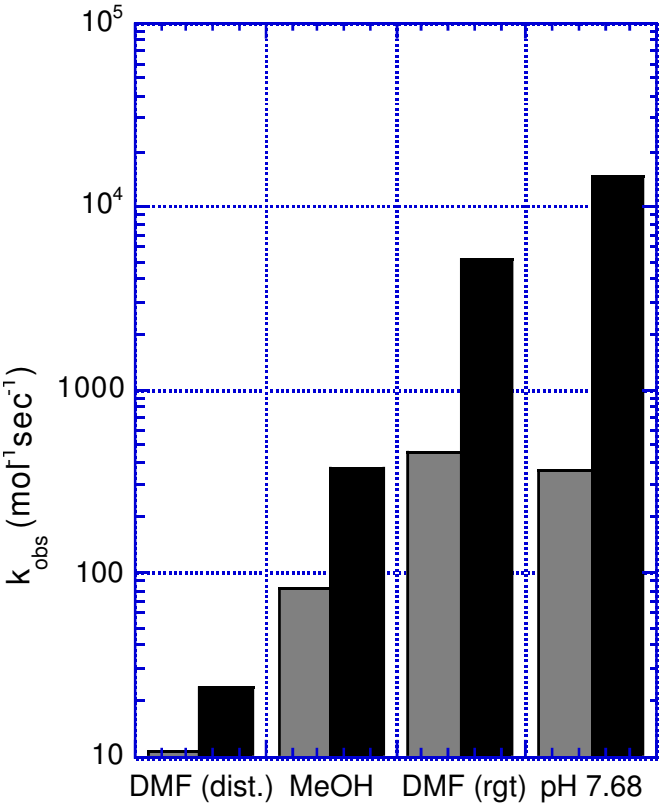

Fig 5. Comparison of k_{obs} for DCIP oxidations of pyranopterin (gray bars) and a tetrahydropterin (black bars) in different solvent media.

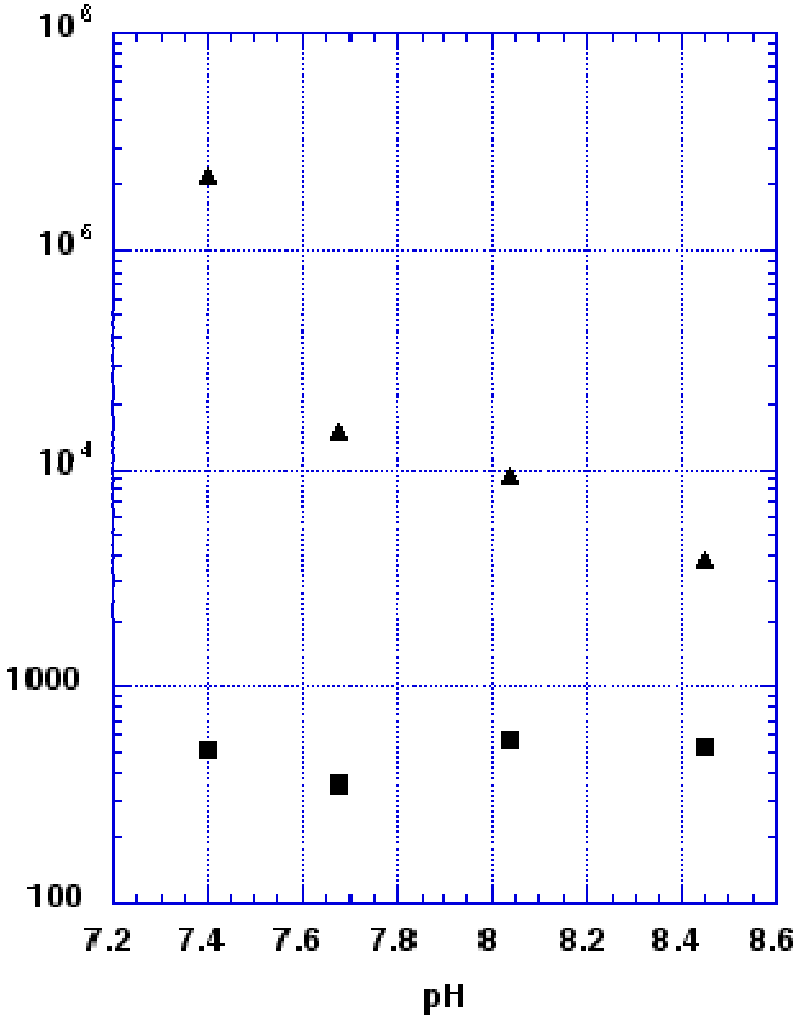

Fig. 6. Comparison of k_{obs} for DCIP oxidations of pyranopterin (squares) and tetrahydropterin (triangles) in TRIS buffer pH 7.4 - 8.5.

Attempted pyranopterin reduction reactions. The possibility for further reduction of pyranopterin **1** from the semi-reduced dihydropterin state to a tetrahydropterin state was investigated using a variety of reductants and methods. Reduction conditions include catalytic heterogeneous hydrogenation, aqueous dithionite and reduced methyl viologen cation radical. In every case, ***no*** reduction was observed.

The well-established synthetic preparation of tetrahydropterins from either fully oxidized or semi-reduced pterins employs catalytic hydrogenation in acidic aqueous or methanolic solution [32]. We anticipated that an acidic environment would readily facilitate pyran ring opening via protonation of the pyran ring oxygen atom and lead to subsequent reduction of pyranopterin **1** to tetrahydroneopterin, as has been suggested elsewhere [20, 33]. However, under various standard catalytic hydrogenation conditions (pH_2 25-40 psi and pH 0-2), no tetrahydropterin was produced and only pyranopterin was recovered and observed by ^{1}H NMR. Subsequent investigation of the stability of the pyran ring in an acidic environment was studied by ^{1}H NMR monitoring of a trifluoroacetic acid solution of pyranopterin **1**. This study confirmed that there is no change nor degradation of pyranopterin over 24 hours in this moderately strong acid.

The ability of methyl viologen radical cation to reduce pyranopterin was tested by titration in TRIS buffer in the range pH 7.0 to 8.3. Methyl viologen radical cation MV^+ was generated by dithionite addition to methyl viologen dichloride. The blue absorption of the methyl viologen radical was monitored as pyranopterin concentration increased. No decrease in the absorbance of the methyl viologen radical cation at 604 nm was observed under any conditions (data not shown) as would be expected if it reduced **1** isolated as hydrochloride salt. In contrast, when the MV^+ titration is performed in unbuffered aqueous solution, a bleaching of the blue color of the methyl viologen radical cation is observed as pyranopterin concentration is increased. This apparent consumption of the MV^+ is not due to pyranopterin reduction. Rather, it was determined to be due to a decrease in pH from the HCl associated with **1**. A control titration of methyl viologen radical cation by HCl at comparable concentrations confirmed the 604 nm bleaching in unbuffered solutions is due to a pH shift.

Discussion

At the time we began this study, there was only one route described in the literature for entry into the fused pyranopteridine system. This route was developed by Pfleiderer et al. whose intention was to investigate the condensation of D and L- arabinose phenyl hydrazones with triaminopyrimidines as a means to obtain further examples of 5,6-dihydropteridines [29]. Their initial characterization of the reaction products by mass spectrometry showed species of the correct mass for the expected dihydropteridines. However, subsequent X-ray crystallography of a 2-methylmercapto derivative proved the products were not 5,6-dihydropteridines but instead had a novel fused pyranopteridine structure resulting from an intramolecular cyclization of the terminal hydroxyl across the pteridine C7-N8 bond (Fig. 3).

The unusual tricyclic structure of the pyranopterin system has been identified by protein crystallography in all X-ray structures of molybdenum and tungsten enzyme possessing the molybdopterin ligand [4,13]. Speculations have been offered as to how the pyranopterin at the catalytic site might couple into the overall redox reactions that are characteristic of Mo and W enzymes. Theoretical evidence for the idea that a ring-opened 5,6-dihydropterin might play a role in the catalytic cycle was supported by recent *ab initio* calculations on a variety of dihydropterin tautomers. The calculation results showed that several tautomers, including 5,6-dihydropterin, were nearly isoenergetic and therefore could reasonably be participants in the catalytic reaction [34]. However, to date there are no quantitative studies on the reactivity of pyranopterin to compare with these conclusions based on computation.

Our study of the oxidative reactivities of the pyranopterin system used oxidants ferricyanide and the redox dye dichloroindophenol (DCIP) for spectrophotomeric titrations of **1 (**Fig. 4)**.** The choice of these two oxidants was based on prior redox titration experiments of molybdopterin in the molybdoenzyme sulfite oxidase [18, 30,31]. That study was the first to call into question the nature of molybdopterin redox state when it concluded that molybdopterin was a dihydropterin, possibly having an unknown structure. These conclusions were based on the observations that the addition of two electron equivalents of either ferricyanide or DCIP produced the observed spectroscopic signature of an oxidized pterin. Our titration experiments confirm that pyranopterin oxidation using either ferricyanide or DCIP is a 2-electron, 2-proton process, i.e., our experiments confirm that pyranopterin behaves as a dihydropterin where pyran ring opening likely precedes formation of an unsaturated oxidized pterin system as depicted in Fig. 2.

The structural relationship between pyranopterin and a tetrahydropterin is emphasized in Fig. 2 and this structural similarity motivated our next goal of comparing the relative oxidative reactivities between pyranopterin and a fully reduced tetrahydropterin. Relative rates of oxidation were obtained from values of observed second order rate constants, k_{obs}, measured under a range of oxidation conditions (Table 1). In every case, tetrahydropterin is kinetically more reactive than pyranopterin towards DCIP oxidation. Furthermore, tetrahydropterin oxidation is strongly dependent on the solvent medium. In the polar aprotic solvent dimethylformamide, the oxidation rate differs by a factor of two whereas in TRIS buffer at pH 7.68 there is a nearly forty-fold difference between the oxidation rates of tetrahydropterin and pyranopterin. In aqueous TRIS buffered solution, the oxidation rate of H_4DMP decreases over sixty-fold with increasing pH between the range pH 7.4 - 8.5. In this same pH range the oxidation rate of pyranopterin **1** is only slightly affected with a minimum rate near neutrality (pH

7.6) . Clearly the superficial similarity of the tetrahydropyrazine structures within pyranopterin and tetrahydropterin are not similarly reactive and pyranopterin is a more robust structure towards oxidants. Having established that pyranopterin reacts as a dihydropterin leads to a question of the relative reactivities of pyranopterin compared to other dihydropterin tautomers. Oxidation of the pyranopterin system in **1** by either DCIP or $[Fe(CN)_6]^{3-}$ is rapid and occurs within minutes. In contrast, 7,8-H_2pterin resists oxidation by oxgyen while the more reactive quinonoid-H_2pterin isomer is oxidized still more slowly than pyranopterin [35].

A dihydro- redox level for pyranopterin implies that further reduction to the tetrahydropterin level should be accessible. A site of unsaturation required for further reduction is exposed if the pyran ring is opened, as illustrated in Fig. 7. Experimental evidence to support the reversibility of the pyran ring opening step is the documented observation of a furanose form which is formed on dissolution of **1** and related dihydroxypyranopteridines (see Fig. 3) [28, 29].

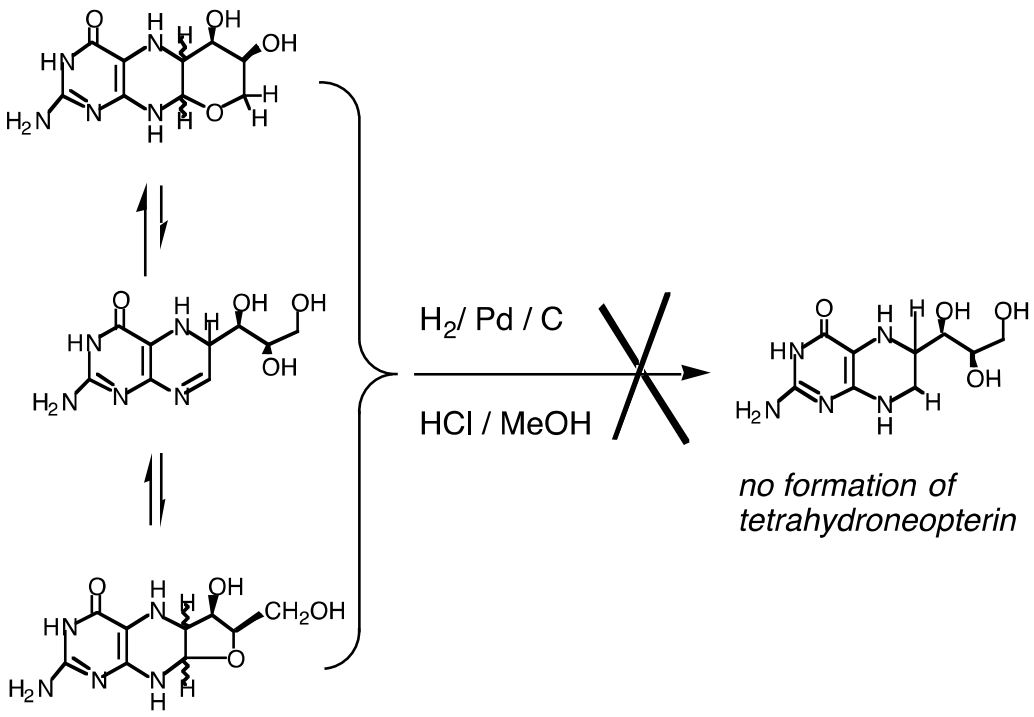

Fig. 7. Pyran ring opening within **1** fails to lead to tetrahydropterin under reducing conditions.

The common method of reducing oxidized pterins to tetrahydropterins is catalytic hydrogenation over Pd/C in aqueous or non-aqueous acidic media [32]. This method applied to pyranopterin **1**, however, fails to form any detectable tetrahydroneopterin. The lack of efficient catalytic reduction of pyranopterin to tetrahydroneopterin indicates that the pyranopterin structure resists further reduction and behaves as a fully reduced pterin. Furthermore, despite the expectation that acidic environments would facilitate pyran ring opening required to expose a site of unsaturation, we observe that pyranopterin is stable towards acidic environments and resists ring-opening.

Other reductants, such as sodium dithionite, methyl viologen cation radical and dithiothreitol are commonly used to maintain anaerobic or reducing environments in biochemical preparations of

molybdenum enzymes and during physical studies on isolated enzyme or Moco cofactor samples [24]. The molybdenum cofactor has typically been isolated in the presence of dithionite to prevent a loss of activity in the Nit-1 assay. Dithionite has been used to reduce the cofactor in protein crystals for crystallography [36,37]. The methyl viologen cation radical ($MV^{\cdot}+$) has a long history as a reductant for biochemical systems and is frequently used to maintain a reducing environment for oxidatively sensitive enzymes and cofactors [38]. We investigated the possible reduction of pyranopterin by dithionite using the redox mediator methyl viologen dication whose concentration can be conveniently monitored using visible spectroscopy. In 50 mM TRIS buffered solutions at pH 7.0, no pyranopterin reduction by dithionite / $MV^{\cdot}+$ is observed. It should be noted that a misleading observation of pyranopterin bleaching blue $MV^{\cdot}+$ species in unbuffered solutions is not due to redox but due to the spectroscopic changes of the ($MV^{\cdot}+$) in acidic solution, as confirmed by a titration of $MV^{\cdot}+$ with HCl. Likewise, experiments with dithiothreitol (DTT) show no detectable reduction in an equimolar mixture of pyranopterin **1** and DTT.

Conclusion

The results from our study illustrate the dual personalities of pyranopterin. In oxidations, it behaves as a dihydropterin to produce a fully oxidized pterin. Under reducing conditions it is unreactive, in other words, it behaves as a fully reduced pterin. The fused pyran ring is key to its unique behavior. While its rapid oxidation by DCIP and ferricyanide classify pyranopterin as a reactive dihydropterin similar to the quinonoid tautomers, the pyran ring blocks rearrangement to the more stable 7,8-dihydro- tautomer in contrast to the facile rearrangements known for the quinonoid tautomers. The pyranopterin is stabilized towards oxidation compared to

tetrahydropterins, despite the similar saturation of their pyrazine rings. The pyran ring is apparently not sufficiently stable once opened and this may be preventing further reduction of the pterin core. On the other hand, once pyranopterin **1** has been oxidized to neopterin, reduction leads to tetrahydroneopterin and does not regenerate the pyran ring.

The work presented here has important implications for understanding the chemistry of the pyranopterin cofactor in molybdenum and tungsten enzymes. Our studies using a relatively simple pyranopterin provide a starting point for delineating what redox reactions are likely within the enzymes. In Fig. 8, the gamut of redox reactions discussed in this paper are illustrated for molybdenum-bound molybdopterin. Addition of the Mo-dithiolene unit to the pyranopterin core can be expected to affect the stabilities of various species shown in Fig. 8, leaving open the questions of which redox reactions and intramolecular rearrangements occur during catalysis within molybdenum and tungsten enzymes. Our results are timely insofar as a recent report of a structure of nitrate reductase A from *E.coli* exhibits one of the two molybdopterin ligands in an open bicyclic form of undetermined pterin redox state [39].

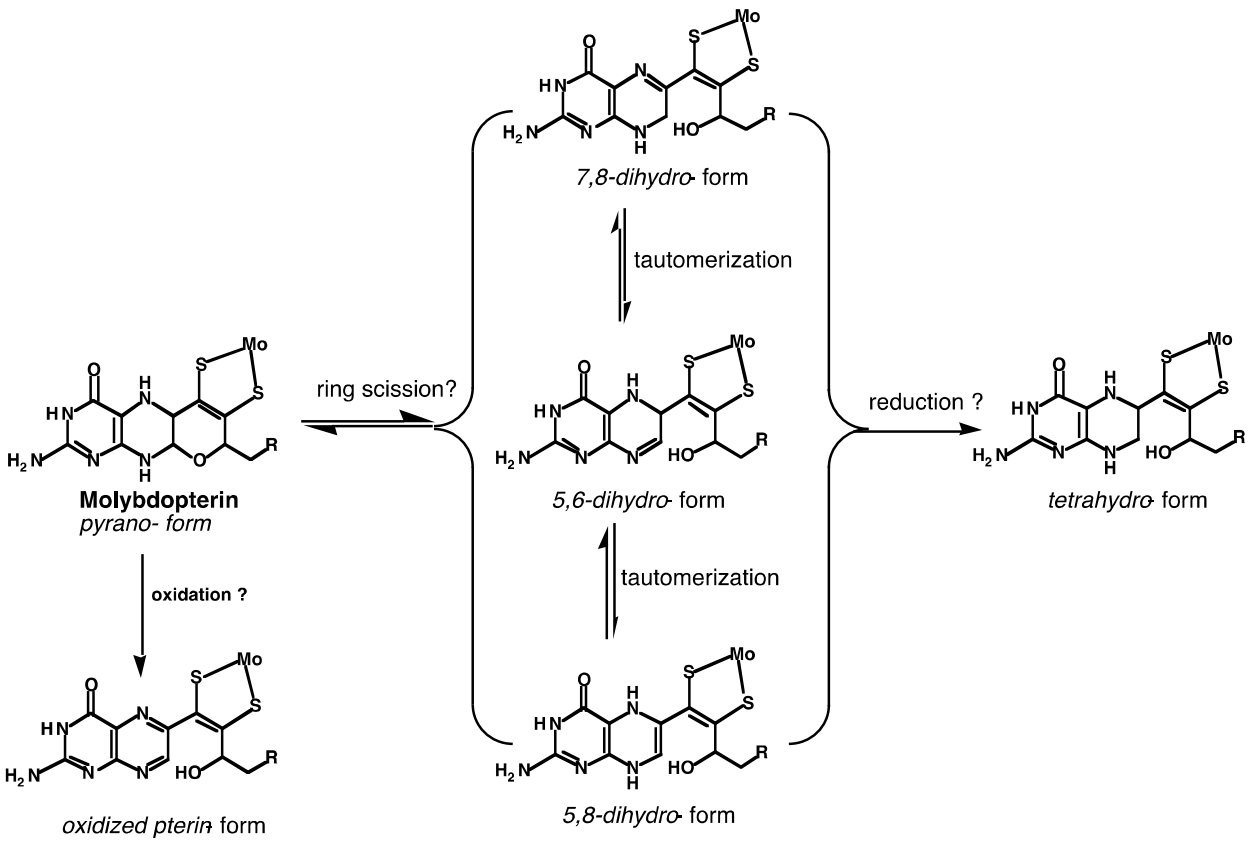

Fig. 8. An illustration of hypothetical redox reactions and intramolecular rearrangements possible for the pyranopterin unit within molybdopterin.

The results of our study provide the first detailed experimental evidence to verify prior interpretations of the molybdenum cofactor as a two electron, two proton reagent in redox reactions. Pyranopterin reactivity as observed in this study indicates those characteristics that might be key to its use in Mo and W enzymes. The pyranopterin system is more stable towards oxidation than tetrahydropterin. The well-established sensitivity of isolated molybdenum cofactor when released from protein can be understood to arise from the irreversible disruption of the pyran ring of molybdopterin. This irreversible pyran ring-opening requires that a highly

protective environment needed during the biosynthesis of the cofactors and transfer to prevent irreversible loss of the pyran functionality [23].

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