

The Tetrahydropyranopterin Structure of the Sulfur-free and Metal-free Molybdenum Cofactor Precursor*

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The molybdenum cofactor (Moco), a highly conserved pterin compound coordinating molybdenum (Mo), is required for the activity of all Mo-dependent enzymes with the exception of nitrogenase. Moco is synthesized by a unique and evolutionary old multi-step pathway with two intermediates identified so far, the sulfur-free and metal-free pterin derivative precursor Z and molybdopterin, a pterin with an enedithiolate function essential for Mo ligation. The latter pterin component is believed to form a tetrahydropyranopterin similar to the one found for Moco in the crystal structure of Mo as well as tungsten (W) enzymes. Here we report the spectroscopic characterization and structure elucidation of precursor Z purified from *Escherichia coli* overproducing MoaA and MoaC, two proteins essential for bacterial precursor Z synthesis. We have shown that purified precursor Z is as active as precursor Z present in *E. coli* cell extracts, demonstrating that no modifications during the purification procedure have occurred. High resolution electrospray ionization mass spectrometry afforded a $[M + H]^+$ ion compatible with a molecular formula of $C_{10}H_{15}N_5O_8P$. Consequently 1H NMR spectroscopy not only allowed structural characterization of the molecule but confirmed that this intermediate undergoes direct oxidation to the previously well characterized non-productive follow-up product compound Z. The 1H chemical shift and coupling constant data are incompatible with previous structural proposals and indicate that precursor Z already is a tetrahydropyranopterin system and carries a geminal diol function in the C1' position.

The molybdenum cofactor (Moco)¹ is part of the active site of all molybdenum (Mo)-dependent enzymes (1) with the exception of nitrogenase and plays important roles in the global carbon, sulfur, and nitrogen cycles (2). Mo enzymes are important for diverse metabolic processes such as sulfur detoxifica-

tion and purine catabolism in mammals (3) as well as nitrogen assimilation and phytohormone synthesis in plants (4). Moco consists of an organic moiety originally called molybdopterin (5), a tetrahydropyranopterin that carries a terminal phosphate group and a Mo atom bound to an enedithiolate system (Fig. 1) (6). In all of the organisms studied so far, Moco is synthesized by an ancient and highly conserved pathway (7). In humans, a mutation in any of the steps of Moco biosynthesis results in the pleiotropic loss of sulfite oxidase, aldehyde oxidase, and xanthine oxidoreductase activity (8, 9). Affected patients show neurological abnormalities and die in early childhood because no therapy is available yet (10). In addition, many prokaryotes, mainly archaea, contain metallo-enzymes with a similar tetrahydropyranopterin enedithiolate cofactor that coordinates W instead of Mo. Therefore, the basic tetrahydropyranopterin component of Mo and W enzymes will be abbreviated as MPT, which stands for molybdopterin or metal-binding tetrahydropyranopterin enedithiolate (11, 12). It is also believed that W cofactor biosynthesis is similar to Moco biosynthesis with the exception of the final metal insertion step (7).

Moco biosynthesis (Fig. 1) can be divided into three steps (7, 13). Initially, the sulfur-free precursor Z is synthesized from a guanosine derivative by the action of two proteins (14, 15). In the second step, precursor Z is converted to MPT by the incorporation of two sulfur atoms at the C1' and C2' positions (see numbering in Fig. 1), forming an enedithiolate function (16). This reaction is catalyzed by the heterotetrameric enzyme MPT synthase (17) that transfers two sulfur atoms from two thio-carboxylated small subunits to the carbon atoms C1' and C2' (18, 19). In the last step of Moco biosynthesis, a Mo atom is transferred to one (prokaryotes and eukaryotes) or two (prokaryotes) MPT enedithiolates, resulting in the formation of Moco (20, 21). In bacteria, an additional modification of the pterin phosphate by nucleotide attachment has been found (22).

The basic chemical structure of Moco was elucidated by the pioneering work of Rajagopalan *et al.* (13, 23). Their final description of Moco was clarified by crystal structures of Mo-(24) and W-containing enzymes (25) detecting the unprecedented observation of an additional pyrano ring, which is formed between the hydroxy group of the C3' atom and the pterin C7 position (Fig. 1). Based on this finding, it was suggested that the tetrahydropyran is either already present in the intermediates (12, 18, 26) or formed during apoenzyme incorporation (27). So far, neither MPT nor precursor Z has been studied by 1H or ^{13}C NMR spectroscopy or crystallized, which leaves the question open whether or not the pterin side chain forms a tetrahydropyranopterin structure in an early stage of the biosynthetic pathway.

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¹ The abbreviations used are: Moco, molybdenum cofactor; Mo, molybdenum; COSY, correlation spectroscopy; HPLC, high pressure liquid chromatography; ESI, electrospray ionization; MS, mass spectrometry; MPT, molybdopterin or metal-binding tetrahydropyranopterin enedithiolate; W, tungsten.

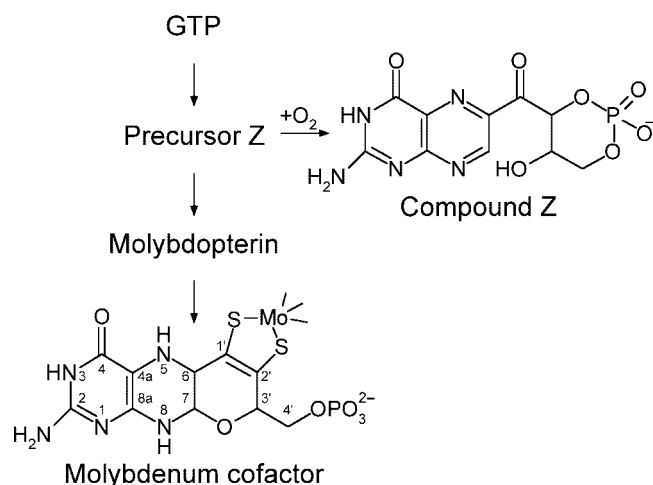


FIG. 1. **General scheme of Moco biosynthesis.** The structures of Moco (as found in the crystal structure of Mo-enzymes) and the precursor Z oxidation product compound Z (14, 15) are shown. Numbering is shown and used as in standard pterin numbering schemes. For a discussion, see Ref. 12.

The structure of precursor Z has been proposed based on the structure of the oxidation product (28), ^{31}P NMR spectroscopy, and mass spectrometry (MS) as well as oxidation studies (14). The direct stoichiometric conversion of precursor Z by a two-electron oxidation to the stable oxidation product compound Z suggested a dihydro-state of precursor Z, which was explained by a quinonoid structure and an enol function in C1' that might be subjected to a keto-enol tautomerization (14). Similar to compound Z, precursor Z is free of sulfur and contains a cyclic phosphate functionality bridging C2' and C4'. In comparison to MPT and Moco, precursor Z is the most stable intermediate with an estimated half-life of several hours at a low pH (14). Therefore, we have established a method for the purification of large amounts of precursor Z derived from *E. coli*. We report a detailed spectroscopic analysis and structural characterization of precursor Z. Using ^1H NMR and MS methods, we demonstrate that precursor Z already possesses a fully reduced tetrahydropyranopterin structure and is predominately hydrated at the C1' position resulting in a geminal diol.

EXPERIMENTAL PROCEDURES

Chemicals—NaCl, yeast, tryptone, and isopropyl- β -D-thiogalactopyranoside were from Duchefa Biochemie; ammonium acetate was from Merck; citric acid monohydrate and sodium citrate dihydrate were from Baker; formic acid was from Sigma; D_2O and DCl were from Deutero GmbH; and NaOD was from Fluka AG.

Construction of Plasmids for Co-expression of MoaA and MoaC—*E. coli moaA* and *moaC* were re-cloned by PCR from pJR11 (29). The published gene sequence (30) was used to design oligonucleotides that permitted cloning into the NdeI and XhoI sites of the multiple cloning region of the pET15b expression vector (Novagen). The resulting plasmids were designated pPH15moaA and pPH15moaC. The complete *moaC* expression unit with the isopropyl- β -D-thiogalactopyranoside-regulatable T7 promoter/operator element, and the synthetic ribosomal binding site of pPH15moaC was then subcloned between the SphI and HindIII sites of pLysS (Novagen), resulting in pPHLysmoaC.

Isolation and Purification of Precursor Z—Precursor Z was purified from *E. coli* using a modified protocol described previously (14). MJ7 *chlM*(DE3) cells (31) containing plasmids pPH15moaA and pPHLysmoaC were grown anaerobically at 20 °C in LB medium supplemented with 120 $\mu\text{g}/\text{ml}$ ampicillin, 30 $\mu\text{g}/\text{ml}$ chloramphenicol, and 50 μM isopropyl- β -D-thiogalactopyranoside and harvested by centrifugation (5 min, 12,000 $\times g$, 4 °C). For standard HPLC purifications, cells were re-suspended in two volumes of 0.4 M HCl, sonicated, and clarified by centrifugation. To prove any modifications that might be caused by the HCl-based acidification, cells were extracted in control experiments with 100 mM acetate buffer, pH 3.0, and processed as described below. The clear supernatant was injected onto a semi-preparative reversed-

phase column (C8, 5 μm , 250 \times 10 mm, Kromasil, EKA Chemicals) equilibrated in 5 mM ammonium acetate, pH 5.0. Precursor Z was eluted in the first absorbing peak and immediately frozen in liquid nitrogen. In the second step, precursor Z-containing samples were pooled and loaded onto a semi-preparative strong anion exchange column (15 μm , 250 \times 10 mm, Adsorbosphere, Alltech) equilibrated with 10 mM citrate buffer, pH 3.0. Precursor Z was eluted isocratically with starting buffer between 20 and 30 ml and frozen in liquid nitrogen. Final purification was achieved by loading the precursor Z onto an analytical reversed-phase column (C18, 5 μm , 250 \times 4 mm, Alltech) equilibrated in 10 mM formic acid. Precursor Z-containing fractions were pooled and frozen in 20- μl aliquots in liquid nitrogen and stored at -80 °C until further use. Precursor Z concentrations were determined using the extinction coefficient of $\epsilon_{267\text{ nm}} = 8960\text{ M cm}^{-1}$ (14).

In Vitro Synthesis of Precursor Z—Precursor Z was converted into molybdopterin using thiocarboxylated *E. coli* molybdopterin synthase (18). The reaction was performed in a 5-ml volume of reaction buffer (100 mM Tris-HCl, pH 7.2) containing either purified precursor Z (160 pmol) or a precursor Z-containing (220 pmol) cell crude extract of MJ7 *chlM*(DE3) cells also used for precursor Z purification and 90 pmol of molybdopterin synthase. The cell extract was freshly prepared by re-suspending 300 mg of *E. coli* cells in 800 μl of reaction buffer, sonication, and clarification. First, the enzyme was diluted in 2.5 ml of reaction buffer. Second, the reaction was started with the addition of 2.5 ml of precursor Z-containing reaction buffer. The reaction was stopped by mixing 400 μl of the reaction mixture with 50 μl of iodine solution (1% I_2 , 2% KI, 1 M HCl). Molybdopterin content was determined by HPLC FormA analysis as described previously (32).

Electrospray Mass Spectrometry—Precursor Z was dissolved in a 1:1 (v:v) methanol, 1% formic acid solution. Approximately, 3 μl of this solution (final concentration of 20 pmol/ μl) were filled into a gold-coated nanospray glass capillary (Protana). The tip of the capillary was placed orthogonally in front of the entrance hole of a quadrupole time-of-flight (Q-TOF 2) mass spectrometer (Micromass) equipped with a nanospray ion source, and a voltage of ~ 1000 V was applied. For collision-induced dissociation experiments, parent ions were selectively transmitted from the quadrupole mass analyzer into the collision cell. Argon was used as the collision gas, and the kinetic energy was set at around -25 eV. The resulting daughter ions were then separated by the orthogonal time-of-flight mass analyzer. The isotopic composition of the sample was determined in the accurate mass mode using reserpine ($[\text{M} + \text{H}]^+ = 609.2811\text{ Da}$) as an internal reference compound.

^1H NMR Spectroscopy—One- and two-dimensional ^1H NMR correlation (COSY) spectra were recorded at 300 K on a Bruker Avance DMX600 NMR spectrometer locked to the major deuterium resonance of the solvent D_2O . Chemical shifts are given in parts/million relative to the residual signal of the solvent (4.80 ppm), and couplings are given in hertz. Precursor Z-containing samples (100–200 μg) were freeze-dried and re-suspended in 700 μl of degassed D_2O and 2 μl of DCl at 36%. To reduce oxidation, nitrogen gas was bubbled into the tube for 5 min. These solutions were sufficiently stable to allow the accumulation of both one-dimensional and two-dimensional COSY spectra. Subsequently, exposure of the solution (400–800 μM in 700 μl) to air (opening the NMR tube) caused oxidation of the precursor to the well documented major single product, compound Z, and this reaction was followed by taking one-dimensional ^1H spectra at regular intervals. Normally, the reaction was completed after 14 days in D_2O and DCl at room temperature.

RESULTS

Isolation and Purification of Precursor Z—Precursor Z was isolated from *E. coli* and purified using a three-step HPLC chromatographic procedure that yielded a homogenous product as proven by UV-visible (data not shown), ESI-MS, and ^1H NMR spectroscopy (see below). An average yield of purified precursor Z was 40 $\mu\text{g}/\text{liter}$ *E. coli* culture with a final concentration of 30–60 $\mu\text{g}/\text{ml}$. The isolated compound was identical with previous preparations (12) because it showed the same UV-visible absorption spectrum with a maximum absorption at 267 nm, pH 3.0, and yielded the same unambiguously characterized oxidation product, compound Z, upon exposure to air (data not shown).

Activity of Purified Precursor Z—To prove the activity of purified precursor Z, we have performed *in vitro* molybdopterin synthesis experiments using recombinant *E. coli* molybdopt-

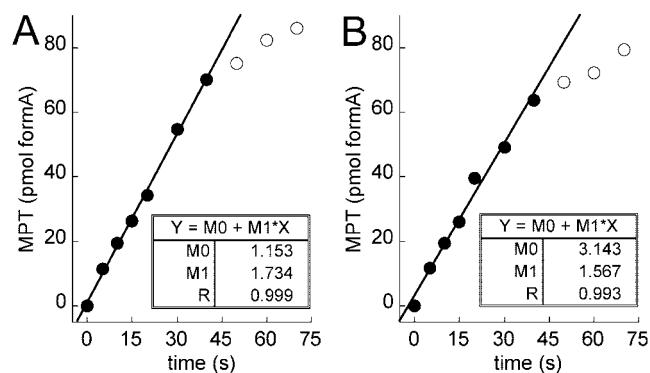


FIG. 2. *In vitro* synthesis of molybdopterin with purified precursor Z (A) and precursor Z present in cell crude extracts (B). 160–220 pmol of precursor Z were converted by 90 pmol of thiocarboxylated *E. coli* molybdopterin synthase (18). Reactions were stopped after 10–70-s reaction times by oxidation in acidic I_2/KI solution. Molybdopterin content was determined by HPLC FormA analysis (32). Values that belong to the linear part of the reaction kinetic (dark dots) were used for regression analysis (inset).

erin synthase (18). The conversion of 160 pmol of precursor Z with 90 pmol of thiocarboxylated molybdopterin synthase followed a linear reaction kinetic over the first 40-s reaction time and reached completion after 1–2 min (Fig. 2A). Because the reaction is stoichiometric, we have observed the formation of 80–85 pmol of molybdopterin. Comparing the reaction curve of purified precursor Z with that of precursor Z (220 pmol) extracted from MJ7 *chlM*(DE3) cells by using a physiological buffer (100 mM Tris-HCl, pH 7.2) revealed an absolutely similar kinetic behavior (Fig. 2B). This finding clearly demonstrates that both purified precursor Z as well as precursor Z present in cell crude extracts are functionally similar in their reaction to molybdopterin, indicating that during the course of purification no modifications have occurred.

ESI-MS of Precursor Z—ESI-MS was employed to determine the molecular mass of precursor Z. Positive ion mode ESI-MS afforded a $[M + H]^+$ ion at 364 Da (Fig. 3A), which was 18 Da higher than the expected mass of 346 Da for the previously proposed structure (14). Indeed there was no evidence for such a peak in the spectrum. A further small peak in the spectrum (Fig. 3A) at 344 Da corresponds to the mass expected for compound Z (14) that is invariably present in such samples because of partial oxidation (Fig. 3A). This fact has also been confirmed by 1H NMR analysis (see below). In contrast to earlier attempts using thermospray or fast atom bombardment ionization, we were able to obtain an accurate mass in the positive ion ESI-MS mode of precursor Z of 364.064 ± 0.003 Da (364.0658 Da calculated for $C_{10}H_{15}N_5O_8P$). Thus, the molecular formula of precursor Z is $C_{10}H_{14}N_5O_8P$, which is incompatible with that proposed previously of $C_{10}H_{12}N_5O_7P$ (12). The new formula of the 364-Da peak suggests a hydration of precursor Z that most probably might be at the C1' position for which a single hydroxylation function with a possible keto-enol tautomerization has been proposed (14). The additional hydration at the C1' would then result in the formation of a geminal diol (Fig. 3A).

MS/MS of the 364-Da peak resulted in the appearance of a major fragment at 166 Da (Fig. 3B), corresponding to a semi-oxidized dihydropterin indicating the pterin nature of that peak. The dihydropterin can be easily formed after tetrahydropyran opening without further oxidation (compare with Moco in Fig. 1). Two other peaks have been observed at 121 and 199 Da, probably corresponding to the masses of the side chain without or with the cyclic phosphate, respectively. Furthermore, MS/MS of the 344-Da peak in the precursor Z sample or MS/MS of purified compound Z afforded a similar daughter ion spectrum with a major fragment at 164 Da (data not shown), corresponding to a

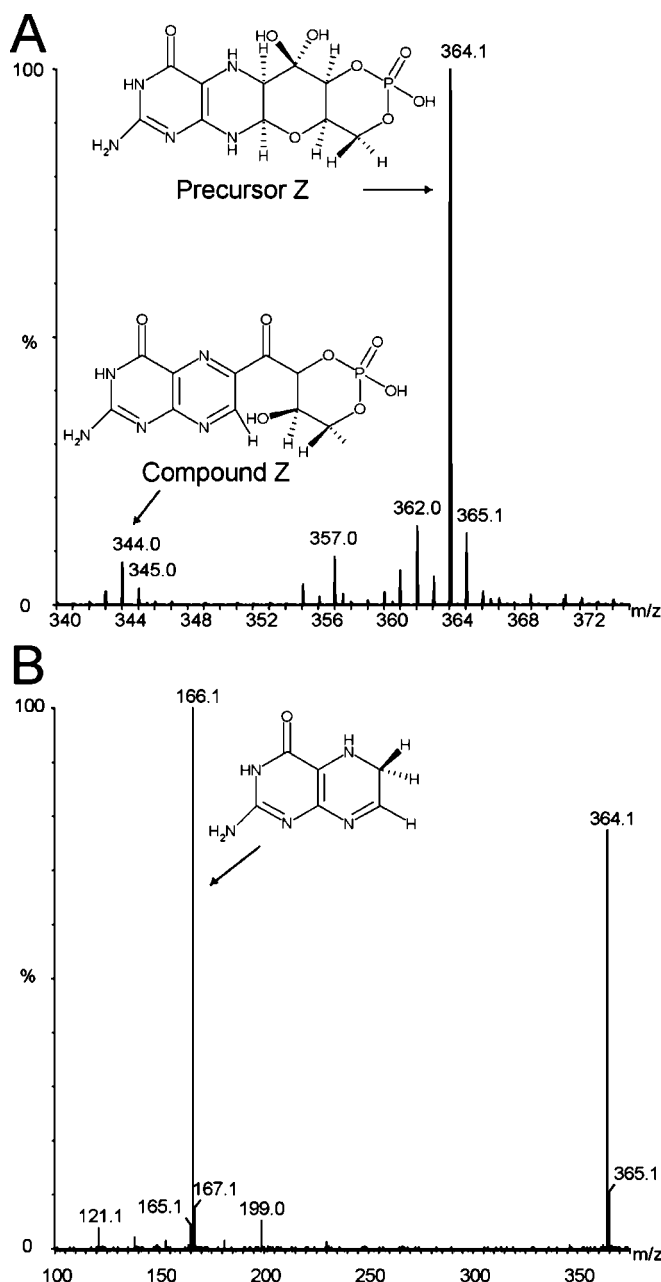


FIG. 3. ESI-MS of precursor Z. A, MS spectrum of purified precursor Z and structural assignment of major molecular ions. B, MS/MS spectrum of the major 364-Da peak shown in A and structural assignment of the major fragment ion. Spectra were recorded in the positive ion mode. Therefore, all of the depicted molecular or fragment ions are shown as neutral compounds and not in the $[M + H]^+$ mode as seen in the spectra.

fully oxidized non-substituted pterin, which is comparable to MS/MS data from other hydrogenated pterins (33).

1H NMR Spectroscopy of Precursor Z—To elucidate the structure of precursor Z and confirm our proposed hydration observed in MS, we have recorded 1H NMR spectra in acidified D_2O and have followed the change of signal positions and intensities during oxidation to compound Z (Fig. 4, A–D). On the basis of 1H chemical shifts (Fig. 4, A–C), connectivities from two-dimensional COSY spectra (Fig. 4D), and the magnitude of coupling constants, all expected signals (six protons, H_a – H_f), were unequivocally assigned (Table I). The 1H chemical shifts and coupling constants (1H – 1H and 31P – 1H) of the oxidation product compound Z were comparable with published data (15, 28) and unambiguously identified this as compound Z (Fig. 4C).

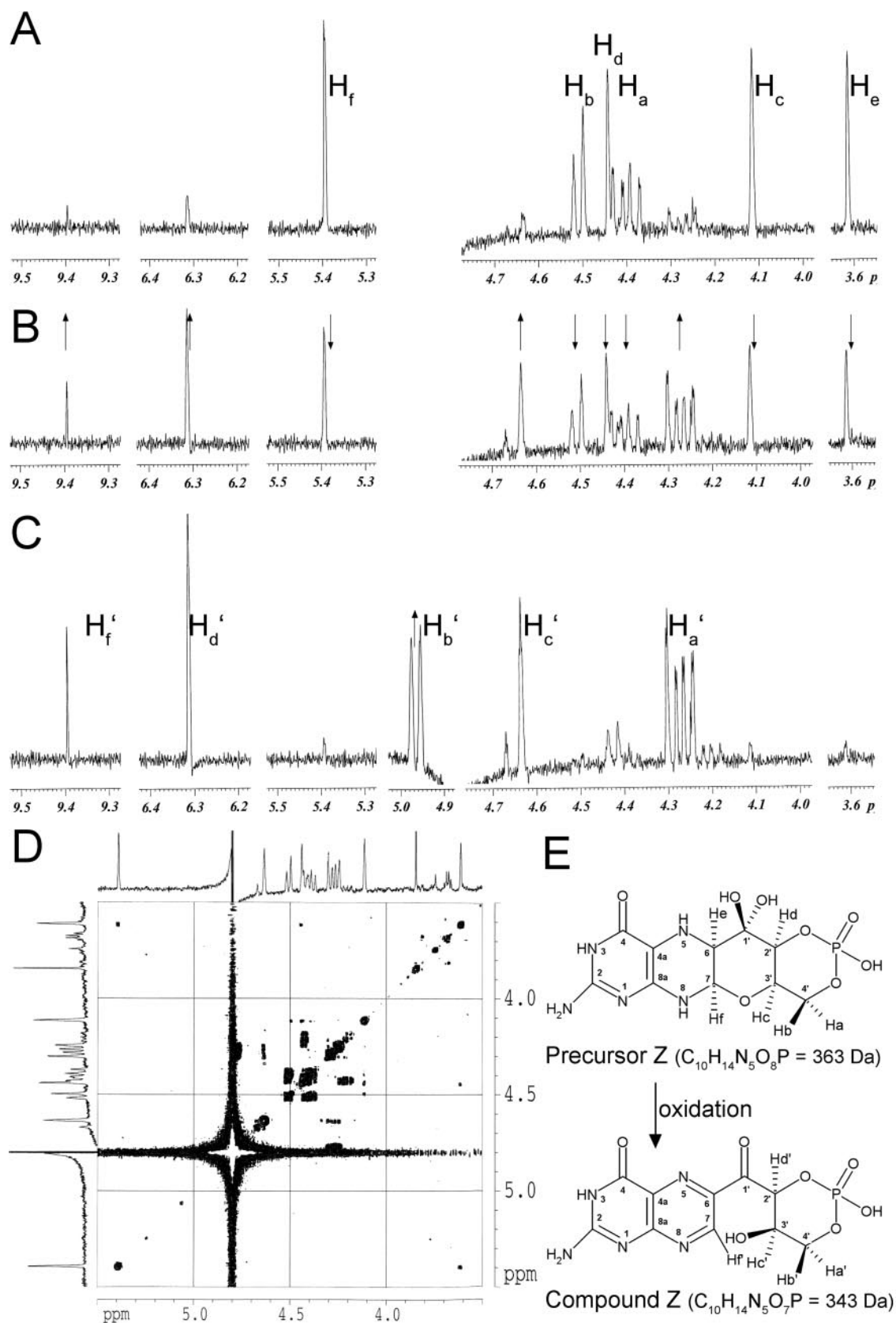


FIG. 4. ^1H NMR and correlation spectroscopy spectra at 300 K of precursor Z of partially oxidized precursor Z and its stable oxidation product compound Z. A–C, the one-dimensional ^1H NMR spectrum of precursor Z was recorded using 150 μg of sample dissolved in 700 μl of D_2O acidified to pH 1 by the addition of 2 μl of DCl at 36%. Measurements were repeated after partial (14 h, B) and complete oxidation (14 days, C). The relevant parts of all three spectra are shown. Note: the doublet of triplets signal for H_f of compound Z is under the residual water signal at 300 K, but it is clearly visible at 315 K (compare 5.0–5.1 ppm in C). D, two-dimensional COSY ^1H NMR spectrum of the partially oxidized precursor Z sample shown in B. E, structure of precursor Z as determined by ESI-MS and ^1H NMR spectroscopy and structure of the oxidation product compound Z. The labeling of the protons is similar to A (precursor Z) and C (compound Z) as well as in Table I and throughout the text. Compound Z protons were marked with a dash to distinguish them from precursor Z.

TABLE I
 ^1H NMR signals of precursor Z and compound Z at pH 1 in $\text{D}_2\text{O}/\text{DCl}$

^1H signals	Chemical shifts ^a ppm	Coupling constants	COSY
		J (^1H - ^1H , ^{31}P - ^1H) H_z	
Precursor Z			
H _a	4.41 (ddd)	22.7 (^{31}P), 13 (H _b), 1.9 (H _c)	H _b , H _c
H _b	4.51 (dt)	13 (H _a), 2.1 (^{31}P), 2.0 (H _c)	H _a , H _c
H _c	4.12 (q) (br)	1.9 (H _a), 1.9 (H _b), 1.9 (^{31}P), 1.9 (H _d)	H _a , H _b , H _d
H _d	4.45 (t) (br)	1.6 (^{31}P), 1.6 (H _c), ~0.8 (H _e)	H _c , H _e
H _e	3.62 (d) (br)	~1.7 (H _f), ~0.8 (H _d)	H _f , H _d
H _f	5.39 (d)	1.7 (H _e)	H _e
Compound Z			
H _a	4.26 (ddd)	22.7 (^{31}P), 12.5 (H _b), 1.9 (H _c)	H _b , H _c
H _b	4.80 (dt)	12.5 (H _a), 1.9 (^{31}P), 1.9 (H _c)	H _a , H _c
	4.97 (dt) at 315 K		
H _c	4.64 (q)	1.9 (H _a), 1.9 (H _b), 1.9 (^{31}P), 1.9 (H _d)	H _a , H _b , H _d
H _d	6.32 (t)	1.6 (^{31}P), 1.6 (H _c)	H _c
H _f	9.39 (s)		

^a Chemical shifts recorded at 300 K if not indicated otherwise. Coupling constants are indicated in parenthesis: s = singlet, d = doublet, t = triplet, q = quintet. Broad peaks are indicated with (br).

The three ^1H NMR signals of protons H_a, H_b, and H_c in precursor Z are comparable with the protons present in the spectrum of compound Z (H_a, H_b, and H_c) in terms of chemical shifts and ^1H - ^1H and ^1H - ^{31}P coupling constants (Fig. 4 and Table I). These three protons were assigned to C4' (H_a and H_b) and C3' (H_c), and the magnitude of the vicinal ^1H - ^{31}P couplings of the C4' protons (22.7 and 2.1 Hz, respectively) were indicative of the intact cyclic phosphate (12). The highest shift of H_c among those three protons indicates significant changes in the environment of C3' that could be explained by the opening of the proposed tetrahydropyranopterin ring structure in precursor Z during oxidation to compound Z.

The signals of the remaining three protons (H_d, H_e, and H_f) of precursor Z differ significantly from those of compound Z (H_d, H_e, and H_f in Fig. 4 and Table I). Precursor Z shows a 1.7-Hz doublet at 5.39 ppm (H_f) that correlates with the broad doublet (broad signal) at 3.62 ppm (H_e). These two signals are gradually lost in the conversion to compound Z with the simultaneous emergence of the signal at 9.39 ppm (H_f in Fig. 4, A-C). The chemical shift of the H_f and H_e signals is comparable with the few pyranopteridines synthesized chemically (34). Independent of the high resolution ESI-MS data, the coupling constants and large difference in chemical shifts of these two protons argues against a previous structure proposal because they would have to belong to a nitrogen-substituted methylene group. The third important signal shows similar vicinal couplings to H_c and P in both precursor Z (H_d) and compound Z but shifts downfield from 4.45 to 6.32 ppm during the oxidation process (Fig. 4 and Table I). These data imply the same relative disposition of H_d with respect to H_c and P in both compounds. The 1.87-ppm difference in the chemical shift of H_d signal strongly favors a significant change in the substituent at C1' between precursor Z and compound Z, which rules out the possibility of the presence of an unchanged keto function in both compounds. Additional ^1H NMR spectra were recorded at different pH values (1, 3, 7, and 10) with no significant changes in proton signals of precursor Z (data not shown). The only noticeable difference at higher pH values was increasing broadness of the H_e and H_f signals accompanied by a faster oxidation to compound Z. Thus, the high resolution ESI-MS and ^1H NMR data for precursor Z, together with changes observed upon oxidation to compound Z, are only compatible with the closed tetrahydropyranopterin structure and a geminal diol in C1' (Fig. 4E). Finally, precursor Z was also extracted under milder conditions (pH 4.0) to exclude a possible chemical modification that could have been a result of acidification (pH 1.5) during cell lysis. Although the overall yield of precursor Z was much lower because of the increased oxidation of precursor Z at

higher pH values, the resulting MS as well as ^1H NMR spectra were identical to those of previous preparations (data not shown).

DISCUSSION

Precursor Z was first identified by Wuebbens and Rajagopalan (14) and attributed to a dihydropterin structure with a C6-substituted 4-carbon side chain carrying a cyclic phosphate attached to C2' and C4' and a hydroxyl function in C1'. Based on the dihydro-state of precursor Z and its relative stability, a quinonoid structure of precursor Z has been suggested with a possible keto-enol tautomerization in the C1' position (14). New insights into the structure of Moco derived from the crystal structure of Mo enzymes (24, 35, 36) raised the question whether or not non-coordinated MPT and/or precursor Z already contain a tetrahydropyranopterin structure (12). In this study, we have addressed this question and verified the side chain structure of precursor Z by applying MS and ^1H NMR spectroscopy to sufficiently large amounts of purified precursor Z. The complete functionality of purified precursor Z has been proven by *in vitro* molybdopterin synthesis that is virtually indistinguishable from the activity of precursor Z present in cell extracts.

One aim of this study was to establish the oxidation state of the precursor Z pterin system to clarify its role in the context of the biosynthetic pathway. The observation of two protons at C6 (H_e) and C7 (H_f) showing a vicinal coupling of 1.7 Hz (COSY) and large chemical shift difference implies that precursor Z is fully hydrogenated in the pyrazine ring of the pterin. A dihydropterin would require a double bond either between N5 and C6 (7,8-dihydro), C6 and C7 (5,8-dihydro), or N8 and C7 (5,6-dihydro). In the case of a 7,8- or 5,8-dihydropterin, the H_e proton would be lost, whereas for the 5,6-dihydropterin, the presence of a double bond between C7 and N8 would result in a substantial shift to a lower field resonance of the H_f signal (~7.5 ppm) because of the withdrawing effect of the double bond as observed in other dihydropterins (37). Furthermore, the chemical shift difference between both pterin protons (H_e and H_f) of 1.77 ppm is very similar to that found in the few reports on chemically synthesized pyranopteridines (H_e = 3.1 ppm, H_f = 4.8 ppm in Me₂SO) (34).

Similarly, it was important to establish whether there is a double bond between C1' and C2' as found in MPT and Moco. The presence of the precursor Z signal at 4.41 ppm (H_d), which reveals the same coupling partners as those found in compound Z, allows the assignment of the proton at the C2' position. Thus, the presence of protons at C2' and C6 ruled out the presence of double bonds either between C6 and C1' (14) or C1'

and C2' (26). Because the ^1H NMR data did not indicate the presence of a proton at C1', it was possible that a carbonyl group was positioned at C1' similar to the one present in compound Z (15, 28). Both the high resolution ESI-MS mass spectrum of precursor Z and the strong difference in chemical shift (1.87 ppm) for the C2'-bound H_d proton between precursor Z and compound Z could not be explained with the aromatization of the pterin ring upon oxidation to compound Z. This shift requires in addition a change in the substituents at C1' with the formation of a geminal diol.

Because all of the ^1H -NMR and ESI-MS spectra were recorded at acidic pH values of 1–3, the observed geminal diol could be the product of an *in vitro* hydration catalyzed under acidic conditions. To see whether a carbonyl group would be formed under different pH conditions in an equilibrium reaction, ^1H -NMR spectra were recorded at different pH values (pH 1–10). The only difference observed in the spectra was the broadening of the signals of H_e and H_f , whereas H_d showed no changes. Hence, we can conclude that the geminal diol is the main product at all of the pH ranges. Hydration at the C1' position of the side chain seems to be a common feature in tetrahydropterins, because an intermediate (6-pyruvoyl-5,6,7,8-tetrahydropterin) in the biosynthesis of tetrahydrobiopterin has been shown to possess a geminal diol at the C1' position with >90% hydroxylation at neutral pH (38). Beside an *in vitro* hydration, the other possibility might be an *in vivo* hydration during the synthesis of precursor Z. Therefore, we suggest that the geminal diol in precursor Z and other reduced pterins might serve a protecting function. So far, the theories proposed for the mechanism of precursor Z biosynthesis (15, 39, 40) do not consider the formation of a geminal diol nor the synthesis of a tetrahydropyranopterin. It has been postulated that precursor Z formation occurs by an alternative cyclohydrolase-like reaction with a guanosine derivative as starting compound (14, 15). During precursor Z biosynthesis, all of the carbon atoms of the guanosine are utilized as the imidazole ring C8 atom is retained and incorporated in a rearrangement reaction as C1' in precursor Z (14, 15). For this reaction, the formation of a transient formyl ester is discussed (15) that might be bound to the conserved and functional important C terminus of proteins of the MOCS1A family (41). The geminal diol might be the product of the formyl release reaction during precursor Z synthesis.

Beside the increase in stability, the geminal diol might serve a second biosynthetic function. We have shown that *E. coli* MPT synthase transfers two sulfur atoms from two different thiocarboxylated small subunits to precursor Z, suggesting the formation of a one-sulfur intermediate (18) whose existence has been demonstrated recently (26). The transfer of the first sulfur is accompanied with the opening of the cyclic phosphate, suggesting the initial attack at the C2' position. The latter makes sense in light of the lower reactivity of the geminal diol at C1' compared with a keto functionality. The second step in the reaction would involve abstraction of both hydroxyl groups and transfer of the second sulfur atom.

In summary, we conclude that the basic structure of precursor Z is a tetrahydropyranopterin. This finding clearly demonstrates that not only Moco but also both intermediates of the biosynthetic pathway (precursor Z and MPT) are present as tetrahydropyranopterins and that the opening of this pyrano ring feature will, with a high probability, irreversibly destroy

all of the intermediates including the cofactor itself. Both pyrano ring formation and the observed geminal diol functionality in C1' seem to be important for (i) protecting precursor Z from oxidation, (ii) maintaining the stereochemistry of the pterin C6 position, and (iii) providing the directed reactivity of precursor Z for MPT synthesis.

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