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Molybdopterin guanine dinucleotide: A modified form of molybdopterin identified in the molybdenum cofactor of dimethyl sulfoxide reductase from *Rhodobacter sphaeroides* forma specialis *denitrificans*

(pterin/5′-GMP)

JEAN L. JOHNSON, NEIL R. BASTIAN, AND K. V. RAJAGOPALAN

Department of Biochemistry, Duke University Medical Center, Durham, NC 27710

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ABSTRACT **The nature of molybdenum cofactor in the bacterial enzyme dimethyl sulfoxide reductase has been investigated by application of alkylation conditions that convert the molybdenum cofactor in chicken liver sulfite oxidase and milk xanthine oxidase to the stable, well-characterized derivative [di(carboxamidomethyl)]molybdopterin. The alkylated pterin obtained from dimethyl sulfoxide reductase was shown to be a modified form of alkylated molybdopterin with increased absorption in the 250-nm region of the spectrum and altered chromatographic behavior. The complex alkylated pterin was resolved into two components by treatment with nucleotide pyrophosphatase. These were identified as di(carboxamidomethyl)molybdopterin and GMP by their absorption spectra, coelution with standard compounds, and by further degradation by alkaline phosphatase to dephospho [di(carboxamidomethyl)]molybdopterin and guanosine. The GMP moiety was sensitive to periodate, identifying it as the 5′ isomer. Chemical analysis of the intact alkylated pterin showed the presence of two phosphate residues per pterin. These results established that the pterin isolated from dimethyl sulfoxide reductase contains the phosphoric anhydride of molybdopterin and 5′-GMP, which is designated molybdopterin guanine dinucleotide.**

The suggestion that all molybdoenzymes contain a common molybdenum cofactor consisting of a low molecular weight organic component complexed to the metal was first put forward in 1964 upon the identification of pleiotropic mutants of *Aspergillus nidulans* that lacked the activities of nitrate reductase and xanthine dehydrogenase (1). In 1971 it was demonstrated that acidified extracts of molybdoenzymes from diverse sources could reconstitute nitrate reductase activity in extracts of a pleiotropic mutant of *Neurospora crassa* deficient in functional molybdenum cofactor (2). A notable exception to the concept of a universal molybdenum cofactor was the demonstration that nitrogenase in fact contains a unique iron-molybdenum cofactor that is not interchangeable with that in other molybdoenzymes (3). The identification of a pterin as the organic component of the molybdenum cofactor, first in sulfite oxidase, xanthine oxidase, and nitrate reductase (4), and later in a host of other molybdoenzymes (5–17), lent further credence to the idea of a common molybdenum cofactor. However, the organic component of the molybdenum cofactor, termed molybdopterin, is extremely unstable when released from its protein environment and has never been structurally characterized in its native state. Instead its structure was deduced from analysis of a number of stable degradation products—form A, form B, and urothione (18, 19)—and was confirmed by specific derivatization of the reactive vicinal sulfhydryl groups producing the dicarboxamidomethyl derivative [di(carboxamidomethyl)]molybdopterin (camMPT) shown in Fig. 1 (20). Generation of camMPT represented a significant advance in the understanding of molybdopterin chemistry. The fact that camMPT can be formed under extremely mild conditions and retains all the structural features of the native pterin (except for oxidation state of the pterin ring) makes it the ideal derivative to probe the chemical identity of the pterin in other molybdoenzymes. Thus, it becomes possible to ask not only whether the cofactor in a given enzyme contains a pterin (i.e., whether it can be degraded to pterin-6-carboxylic acid, form A or form B, or some uncharacterized fluorescent derivative) but to pinpoint whether that pterin is in fact a molybdopterin derivative.

In this paper, we report the results of alkylation of the molybdenum cofactor in the bacterial enzyme dimethyl sulfoxide (DMSO) reductase. Isolation and characterization of the alkylated pterin has revealed the presence of molybdopterin in pyrophosphate linkage to the nucleotide 5′-GMP (see Fig. 1). Isolation of this variant of molybdopterin, designated molybdopterin guanine dinucleotide, by analogy to flavin adenine dinucleotide and nicotinamide adenine dinucleotide, indicates that the pterin-containing cofactor can exist in at least two very different structural forms and raises important questions as to the distribution and functional significance of the two forms.

EXPERIMENTAL PROCEDURES

Rhodobacter sphaeroides forma specialis *denitrificans* IL106, kindly provided by T. Satoh (Tokyo Metropolitan University), was cultured and harvested as described by Satoh and Kurihara (21). The DMSO reductase was purified essentially as described (21). For isolation of [di(carboxamidomethyl)]molybdopterin guanine dinucleotide (camMGD), 40 mg of enzyme in 20 ml of 10 mM sodium phosphate (pH 7.0) was combined with 80 mg of iodoacetamide and the solution was made anaerobic in a serum vial. The enzyme was reduced by the addition of 100 μl of 100 mM sodium dithionite and then denatured by the addition of 200 mg of SDS. The mixture was incubated in the anaerobic vial at 37°C for 2 hr and then at room temperature overnight. The sample was subjected to ultrafiltration using an Amicon stirred cell with a PM-10 membrane. Deistung and Bray (22) have shown that the ultrafiltration step is an effective means of resolving pterin from denatured protein and from a significant portion

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Abbreviations: MGD, molybdopterin guanine dinucleotide; camMGD, [di(carboxamidomethyl)]molybdopterin guanine dinucleotide; camMPT, [di(carboxamidomethyl)]molybdopterin; DMSO, dimethyl sulfoxide.

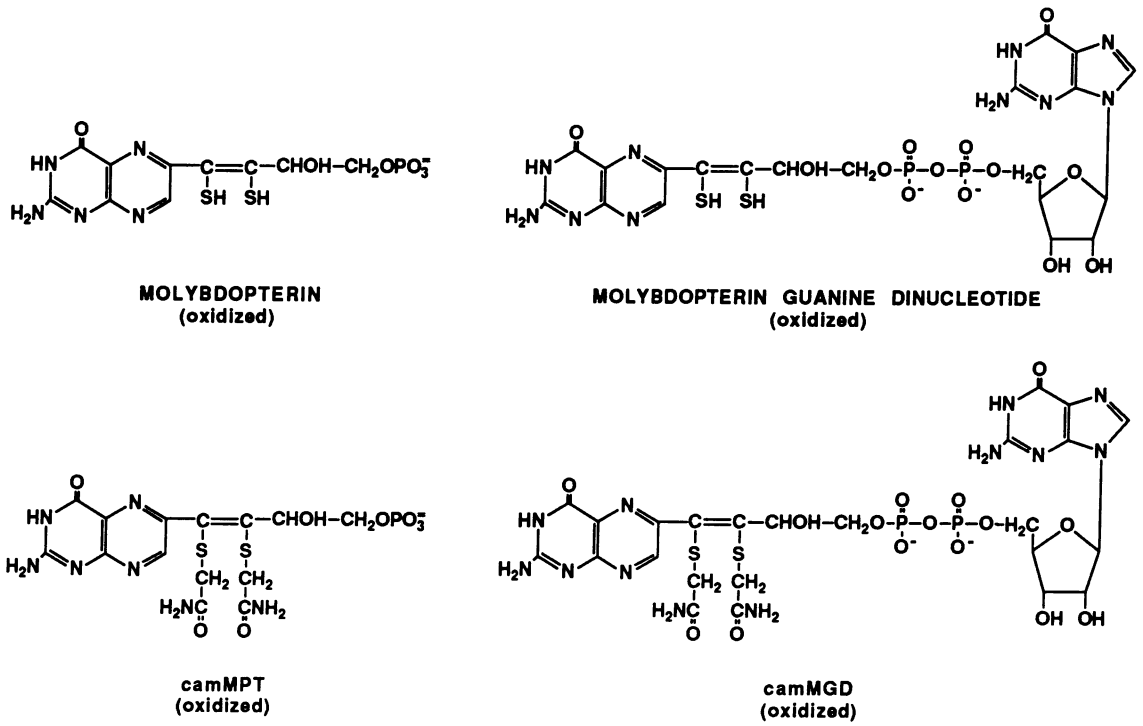

FIG. 1. Structures of oxidized forms of molybdopterin, camMPT, MGD, and camMGD.

of the detergent as well. The Amicon effluent was then diluted 1:2 with water and applied to a 4-ml column of QAE-Sephadex (acetate form) equilibrated with water. The column was washed with 25 ml of water, 50 ml of 10 mM acetic acid, and finally with 200 ml of 10 mM HCl. Fractions containing camMGD were identified by absorption spectroscopy, pooled, neutralized with NH_4OH, and concentrated by rotoevaporation. Final purification of camMGD was achieved by chromatography on a C-18 HPLC column in 50 mM ammonium acetate (pH 6.8) containing 3% (vol/vol) methanol. The same procedure was used to isolate camMPT from chicken liver sulfite oxidase and bovine milk xanthine oxidase purified in this laboratory (23, 24).

HPLC was carried out with a Hewlett–Packard 1090 liquid chromatograph equipped with a 1040A diode array detector. A 10-μm C-18 column (4.6 × 250 mm) supplied by Alltech Associates was used. Absorption spectra were obtained on-line during chromatographic separations and were also evaluated by using a Shimadzu UV-265 spectrometer. Phosphate analysis was carried out according to the procedure of Ames (25).

HPLC grade ammonium acetate and methanol, sodium dithionite, and sodium periodate were obtained from Fisher. Electrophoresis purity SDS was from Bio-Rad. Iodoacetamide, 5′-GMP, guanosine, nucleotide pyrophosphatase (type III), and QAE-Sephadex were from Sigma. Alkaline phosphatase (chicken intestine) was supplied by Worthington.

RESULTS

Identification of a Modified Form of Alkylated Molybdopterin (camMGD). Repeated attempts at isolation of camMPT from purified DMSO reductase using the procedure routinely applied to chicken liver sulfite oxidase and bovine milk xanthine oxidase (20) failed to produce any alkylated product with the elution properties of camMPT. The published procedure for isolation of camMPT makes use of DMSO as a protein denaturant to release the alkylated product from the enzyme. Recognizing that DMSO may not be an equally effective denaturant of all molybdoenzymes, an alternative release procedure using the universal protein denaturant SDS was devised. The procedure, outlined above, proved to be very effective for isolation of camMPT in good yield from both sulfite oxidase and xanthine oxidase. Again, however, identical treatment of a preparation of DMSO reductase failed to yield any camMPT in the expected fractions. Absorption spectra of individual fractions eluting from QAE-Sephadex clearly showed the presence of camMPT from xanthine oxidase or sulfite oxidase in those fractions corresponding to the pH transition between 3.5 (10 mM acetic acid) and pH 2.1 (10 mM HCl). In preparations of DMSO reductase, no oxidized pterin was detected in any of the fractions. However, fractions eluting well after the pH transition contained material with an absorption maximum near 260 nm, which was converted upon aerobic incubation overnight at room temperature to a species with a spectrum suggestive of an oxidized pterin different from that of camMPT. HPLC chromatography of material in these fractions on a C-18 column revealed the presence of a pterin-like material that eluted much later with 50 mM ammonium acetate as mobile phase, 29.5 min vs. 10.4 min for standard camMPT. The absorption spectra of the compound obtained from DMSO reductase and of camMPT from sulfite oxidase are shown in Fig. 2. Both pterin derivatives display the long wavelength band at 367 nm with nearly complete identity in shape. The notable difference between the two is the presence of an additional strong absorption band at 250 nm in the pterin from DMSO reductase. This additional UV-absorbing component was presumably responsible for the increased hydrophobicity of the pterin from DMSO reductase on reverse-phase HPLC.

Identification of camMPT and GMP as Degradation Products of camMGD. A significant clue to the chemical nature of the product was obtained when fractions from QAE-Sephadex containing the material were pooled and concentrated by rotoevaporation. The camMPT derived from sulfite oxidase or xanthine oxidase is quite stable to rotoevaporation at temperatures of 37°C or below with the only degradation being the formation of very small amounts of deamidated products if the solution is not neutralized before evaporation.

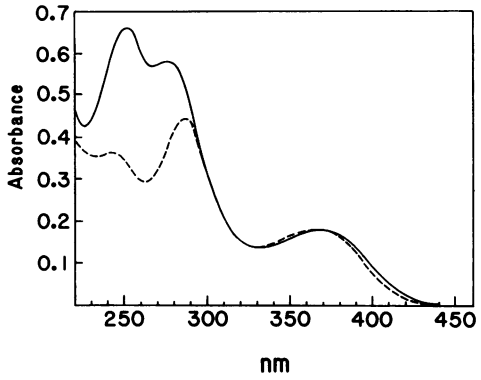

FIG. 2. Absorption spectra of camMGD (—) and camMPT (---) in 50 mM ammonium acetate (pH 6.8).

The alkylated derivative from DMSO reductase, in contrast, was nearly completely destroyed by this treatment, presumably by the brief exposure to the high concentration of HCl generated during rotoevaporation. Reverse-phase chromatography of the concentrated sample revealed that it now contained two major species, one with the elution behavior and absorption spectrum identical to those of camMPT and the other eluting earlier and displaying an absorption maximum at 250 nm. Some minor peaks were also present corresponding to the deamidated camMPT derivatives. A comparison of the absorption properties of the 250-nm absorbing material to those of known low molecular weight biological compounds revealed a strong similarity to those of guanine and its nucleoside and nucleotides. It appeared from these results that the second component present in the intact pterin isolated from DMSO reductase could be a guanine derivative, possibly GMP linked to molybdopterin by the acid-labile phosphoric anhydride bond.

The proposed pyrophosphate linkage should be susceptible to enzymatic cleavage by nucleotide pyrophosphatase, which hydrolyzes the analogous bonds in FAD and NAD. The DMSO reductase pterin was, in fact, very sensitive to the pyrophosphatase and was converted to the same two products formed by acid treatment. Large quantities of the two constituent parts were readily prepared by pyrophosphatase treatment to cleave the phosphoric anhydride bond and reverse-phase chromatography to resolve the two products. The absorption spectra of the products obtained thus are shown in Fig. 3 along with those of authentic GMP and camMPT.

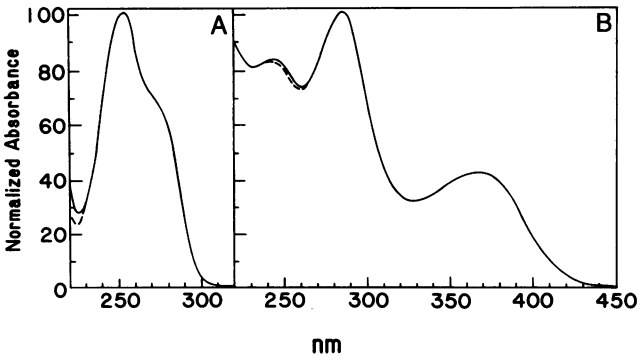

FIG. 3. Absorption spectra of the two degradation products of camMGD produced by pyrophosphatase cleavage. (*A*) Spectrum of the guanine nucleotide product of camMGD (—) compared with that of authentic 5′-GMP (---). (*B*) Spectrum of the pterin product of camMGD (—) compared with that of standard camMPT (---). All spectra were recorded in 50 mM ammonium acetate (pH 6.8).

The products of pyrophosphatase cleavage of the modified alkylated pterin were further identified as GMP and camMPT by HPLC coelution with the standard compounds as shown in Fig. 4 *A–C*. Finally, as anticipated, alkaline phosphatase

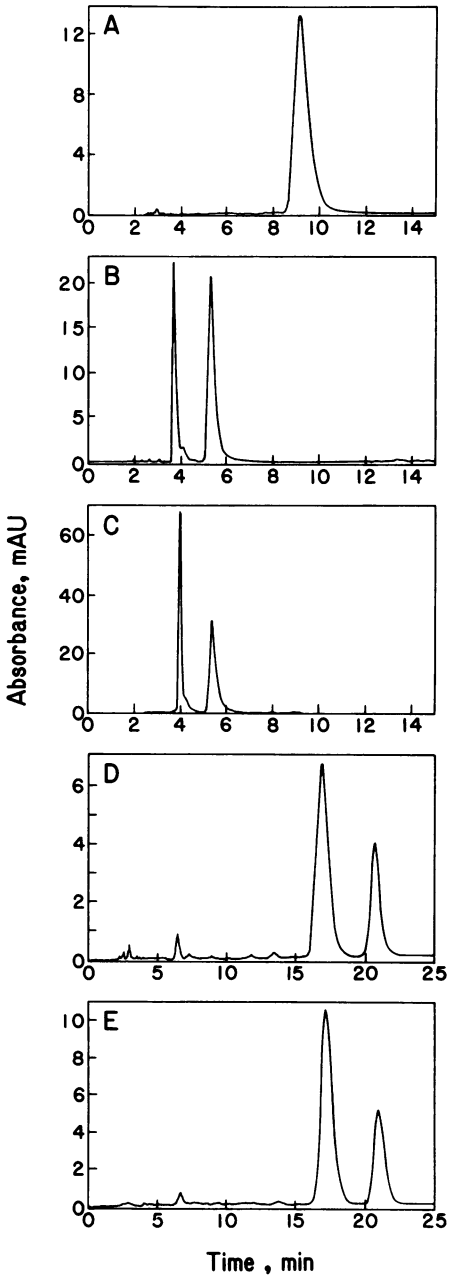

FIG. 4. HPLC elution profiles of camMGD (*A*) and its degradation products (*B–E*). The sample in *B* was incubated with $MgCl_2$ and nucleotide pyrophosphatase for 10 min prior to chromatography. The material eluting at ≈4 min has an absorption spectrum corresponding to that of 5′-GMP, while that at 5.5 min has a spectrum identical to that of camMPT. (*C*) Chromatogram obtained when the pyrophosphatase-treated sample was combined with standard 5′-GMP and camMPT prior to injection. The sample in *D* was treated with $MgCl_2$ and nucleotide pyrophosphatase as in *B* and then with alkaline phosphatase. The material eluting at 17 min has an absorption spectrum corresponding to that of dephospho-camMPT (spectrally indistinguishable from camMPT) and that at 21 min has a spectrum identical to that of guanosine (spectrally indistinguishable from GMP). (*E*) Chromatogram obtained when the sample treated with pyrophosphatase and alkaline phosphatase was combined with standard guanosine and dephospho-camMPT prior to injection. Chromatography in all cases was on a C-18 column in 50 mM ammonium acetate (pH 6.8) containing 3% methanol. mAU, absorbance units × 10^{-3}.

treatment of the pyrophosphatase-cleaved mixture generated two products that coeluted with guanosine and dephospho-camMPT (Fig. 4 *D* and *E*). It should be noted that low levels of alkaline phosphatase, sufficient to dephosphorylate GMP and camMPT in 2 hr, had no effect on the intact pyrophosphate-containing pterin over the same time period. However, 500-fold higher levels of alkaline phosphatase did convert the intact pterin to guanosine and dephospho-camMPT. These results suggest that the alkaline phosphatase preparation contains a weak pyrophosphatase activity, either as a contaminating protein or as a minor activity of the phosphatase enzyme itself.

Identification of the Guanosine Derivative as 5′-GMP. The remarkable similarity in absorption spectra and coelution of the pyrophosphatase product of camMGD with 5′-GMP left little doubt that the appended component was GMP. However, the position of the phosphate on the ribose ring was not defined by the chromatography conditions used, which might not resolve 5′-, 2′-, and 3′-GMP. However, among these, 5′-GMP is the only isomer susceptible to cleavage by periodate. Treatment of authentic 5′-GMP with 0.1 mM periodate at pH 4.5 and analysis of the reaction mixture by HPLC revealed that the nucleotide was converted over the course of 15 min to a product whose absorption spectrum was unchanged but that eluted 2 min earlier from the C-18 column. Identical behavior was observed upon periodate treatment of the GMP derived from the camMGD establishing it as the 5′-isomer.

Phosphate Analysis of camMGD. The results presented above indicated that camMGD contains at least two phosphate residues per pterin. Chemical analysis of phosphate content of intact camMGD was necessary, however, to determine whether additional residues were present that were not detected by analysis of the degraded products. Quantitation of camMGD for phosphate analysis was based on its absorption properties. It was observed that the absorption spectrum of camMGD corresponded very closely to the sum of the spectra of camMPT and GMP. Pyrophosphatase cleavage of camMGD did not produce significant alterations in the absorption spectrum of the unresolved mixture of products, and computer subtraction of a camMPT spectrum from that of camMGD (matched by the absorbance at 367 nm) produced an excellent simulation of the GMP spectrum. From these results, it was apparent that camMPT and camMGD have the same extinction coefficient at 367 nm. For accurate quantitation, samples of camMPT and camMGD prepared in parallel were analyzed for organic phosphate. The molar extinction coefficient for camMPT in ammonium acetate (pH 6.8) corresponding to a phosphate content of one per pterin was calculated to be 7340 at 367 nm. When this value was used, camMGD was found to contain 2.03 mol of phosphate per mol. It should be noted that the value of 7340 for oxidized camMPT is significantly different from that reported in an earlier publication (20). Verification that 7340 is the correct extinction coefficient was obtained by a quantitative comparison of the camMPT and camMGD spectra at 252 nm. As shown in Table 1, the extinction coefficient of camMGD at this wavelength corresponds very closely to the mathematical sum of the contributions of camMPT and GMP.

Table 1. Extinction coefficients of camMPT and camMGD in ammonium acetate (pH 6.8) based on organic phosphate content

	ε, M^{-1}	
	At 367 nm	At 252 nm
camMPT	7340	13,430
5′-GMP	0	13,700*
camMPT + 5′-GMP	7340	27,130
camMGD	7340	26,890

*From ref. 26.

DISCUSSION

The DMSO reductase from *Rhodobacter sphaeroides* is unusual among molybdoenzymes in that it contains the molybdenum cofactor as its single prosthetic group. The pterin nature of the cofactor has been documented in an earlier publication (21) by demonstration of the generation of a fluorescent material characteristic of the form B derivative under appropriate conditions. The studies described here have demonstrated that significantly more structural information can be obtained by application of mild alkylation conditions to stabilize the labile enedithiol of the molybdopterin side chain. By this approach, we have shown that the alkylated pterin isolated from DMSO reductase indeed contains molybdopterin, but in covalent association with 5′-GMP.

The identification of this extended form of the pterin has borne out an earlier speculation that in fact modified forms of molybdopterin could exist (4) and also sheds some light on the nature of an unusual pterin identified but not structurally characterized in carbon monoxide dehydrogenase from *Pseudomonas carboxydoflava* (27–29). Krüger and Meyer have presented evidence suggesting that the enzyme contains a pterin cofactor larger than molybdopterin, bearing two phosphate residues, and cleaved into a fluorescent phosphorylated pterin and a nonfluorescent phosphorylated aromatic component by heating in the presence of perchloric acid. The pterin component was partially characterized by conversion to form A and form B (8) and to pterin-6-carboxylic acid (29) under appropriate conditions and from evidence suggesting the presence of a thiol group on the molecule (29). No information was presented on the identity of the second component, and the nature of the association of the two components was unclear in view of the conclusion (28, 29) that both phosphate residues were monoesters (removed by alkaline phosphatase). It now appears possible that the pterin studied by Krüger and Meyer is derived from molybdopterin guanine dinucleotide (MGD), but additional experiments will be required to establish this with certainty.

Krüger and Meyer have proposed the term "bactopterin" to describe the larger pterin variant that they have noted to be present in various molybdoenzymes of bacterial origin (27, 28, 30). It would seem that at this point such a designation may be premature. An exciting possibility yet to be investigated is that bacteria and eukaryotes may both select molybdopterin for function in certain enzymes and MGD for others, much as FAD and FMN are found in both prokaryotic and eukaryotic enzymes. Further studies will be required to probe the distribution in nature of the two molybdopterin variants thus far described and indeed to ascertain whether yet others exist.

An additional question raised by these studies concerns the function of the GMP component in MGD. Its possible importance in cofactor–protein association or in modulating the redox potential of the pterin and/or metal of the molybdenum cofactor is yet to be addressed. It has been reported that the apoprotein of nitrate reductase present in extracts of *N. crassa nit-1* is reconstituted with equal efficiency using pterin from carbon monoxide dehydrogenase and xanthine oxidase (27). It should now be possible to evaluate these results more critically to determine whether one of the pterin derivatives is modified by enzymes present in the *Neurospora* extract (MGD cleaved to molybdopterin by a pyrophosphatase or molybdopterin activated to MGD) before incorporation or whether either pterin can bind and catalyze the reduction of nitrate. There is some indication that the pterin present in

carbon monoxide dehydrogenase is more resistant to air oxidation than is reduced molybdopterin (27), and indeed in our studies camMGD was first recognized as a 260-nm absorbing reduced pterin in fractions eluting from QAE-Sephadex, whereas camMPT was eluted in its oxidized form. However, more systematic evaluation of the oxidation potentials of the isolated pterin derivatives is clearly warranted.

Finally, these results open a new area of study with regard to the biosynthesis of MGD. Characterization of the enzyme system that presumably converts molybdopterin to MGD and identification of the chemical nature of the guanine nucleotide used as cosubstrate should reveal additional interesting facets of molybdopterin biochemistry.

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1. Pateman, J. A., Cove, D. J., Rever, B. M. & Roberts, D. B. (1964) *Nature (London)* **201,** 58–60.
2. Nason, A., Lee, K.-Y., Pan, S.-S., Ketchum, P. A., Lamberti, A. & DeVries, J. (1971) *Proc. Natl. Acad. Sci. USA* **68,** 3242–3246.
3. Pienkos, P. T., Shah, V. K. & Brill, W. J. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5468–5471.
4. Johnson, J. L., Hainline, B. E. & Rajagopalan, K. V. (1980) *J. Biol. Chem.* **255,** 1783–1786.
5. Schauer, N. L. & Ferry, J. G. (1982) *J. Bacteriol.* **150,** 1–7.
6. Yamamoto, I., Saiki, T., Lin, S.-M. & Ljungdahl, L. G. (1983) *J. Biol. Chem.* **258,** 1826–1832.
7. Dilworth, G. L. (1983) *Arch. Biochem. Biophys.* **221,** 565–569.
8. Meyer, O. & Rajagopalan, K. V. (1984) *J. Bacteriol.* **157,** 643–648.
9. Ishizuka, M., Toraya, T. & Fukui, S. (1984) *Biochim. Biophys. Acta* **786,** 133–143.
10. Liu, C.-L. & Mortensen, L. E. (1984) *J. Bacteriol.* **159,** 375–380.
11. Redinbaugh, M. G. & Campbell, W. H. (1985) *J. Biol. Chem.* **260,** 3380–3385.
12. Siefermann-Harms, D., Fritz, B. & Ninnemann, H. (1985) *Photochem. Photobiol.* **42,** 771–778.
13. Yamamoto, I., Shimizu, H., Tsuji, T. & Ishimoto, M. (1986) *J. Biochem.* **99,** 961–969.
14. Yamamoto, I., Okubo, N. & Ishimoto, M. (1986) *J. Biochem.* **99,** 1773–1779.
15. Fukuoka, M., Fukumori, Y. & Yamanaka, T. (1987) *J. Biochem.* **102,** 525–530.
16. Seki, S., Hattori, Y., Hasegawa, T., Haraguchi, H. & Ishimoto, M. (1987) *J. Biochem.* **101,** 503–509.
17. Weiner, J. H., MacIsaac, D. P., Bishop, R. E. & Bilous, P. T. (1988) *J. Bacteriol.* **170,** 1505–1510.
18. Johnson, J. L., Hainline, B. E., Rajagopalan, K. V. & Arison, B. H. (1984) *J. Biol. Chem.* **259,** 5414–5422.
19. Johnson, J. L. & Rajagopalan, K. V. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 6856–6860.
20. Kramer, S. P., Johnson, J. L., Ribeiro, A. A., Millington, D. S. & Rajagopalan, K. V. (1987) *J. Biol. Chem.* **262,** 16357–16363.
21. Satoh, T. & Kurihara, F. N. (1987) *J. Biochem.* **102,** 191–197.
22. Deistung, J. & Bray, R. C. (1989) *Biochem. J.* **263,** 477–483.
23. Kessler, D. L. & Rajagopalan, K. V. (1972) *J. Biol. Chem.* **247,** 6566–6573.
24. Rajagopalan, K. V. (1985) in *CRC Handbook of Methods for Oxygen Radical Research*, ed. Greenwald, R. A. (CRC, Boca Raton, FL), pp. 21–23.
25. Ames, B. E. (1966) *Methods Enzymol.* **8,** 115–118.
26. Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M. (1986) *Data for Biochemical Research* (Clarendon, Oxford), p. 109.
27. Krüger, B. & Meyer, O. (1986) *Eur. J. Biochem.* **157,** 121–128.
28. Meyer, O., Jacobitz, S. & Krüger, B. (1986) *FEMS Microbiol. Rev.* **39,** 161–179.
29. Krüger, B. & Meyer, O. (1987) *Biochim. Biophys. Acta* **912,** 357–364.
30. Krüger, B., Meyer, O., Nagel, M., Andreeson, J. R., Meincke, M., Bock, E., Blumle, S. & Zumft, W. G. (1987) *FEMS Microbiol. Lett.* **48,** 225–227.