Identification of Molybdopterin as the Organic Component of the Tungsten Cofactor in Four Enzymes from Hyperthermophilic Archaea*

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The hyperthermophilic Archaea represent some of the most ancient organisms on earth. A study of enzymatic cofactors in these organisms could provide basic information on the origins of related cofactors in man and other more recently evolved organisms. To this end, the nature of the tungsten cofactor in aldehyde ferredoxin oxidoreductases from Pyrococcus furiosus and ES-4 and in formaldehyde ferredoxin oxidoreductases from P. furiosus and Thermococcus litoralis has been investigated. All four proteins contain molybdopterin, previously characterized as the organic component of the molybdenum cofactor in a large number of molybdoenzymes. Molybdopterin was identified by conversion to the dicarboxamidomethyl derivative by alkylation of the vicinal sulfhydryl groups on the pterin side chain and by conversion to the oxidized fluorescent derivative, Form A. The pterin of the tungsten cofactor in the four enzymes was examined for the presence of appended GMP, CMP, AMP, or IMP previously observed in molybdenum cofactors of some molybdoenzymes. No evidence for the presence of a molybdopterin dinucleotide or other modified form of molybdopterin was obtained. These results further document the essential nature of molybdopterin for the function of molybdenum and tungsten enzymes in diverse life forms.

The hyperthermophilic Archaea, formerly archaeabacteria (1), are a recently discovered group of microorganisms that have the remarkable property of growing optimally at temperatures near and even above 100 °C (2–4). They occupy a unique ecological niche near undersea hydrothermal vents. Such an unusual growth environment would suggest the evolution of unusual survival mechanisms to maintain cellular structure and to carry out metabolic reactions at extreme temperatures. For example, enzymatic function might require the development of modified protein structures and perhaps the incorporation of unusual prosthetic groups. However, this appears not to be the case with small redox proteins as the recent elucidation of the three-dimensional structure of a hyperthermophilic rubredoxin, using both NMR and x-ray crystallography (5, 6), showed that this protein is stabilized by conventional intraprotein interactions.

On the other hand, recent studies have shown that the growth of several hyperthermophilic organisms requires tungsten, an element seldom used in biological systems (7–10). In Pyrococcus furiosus (11), which grows optimally at 100 °C, tungsten (and no molybdenum) is found in two different iron-sulfur-containing enzymes, which are termed aldehyde ferredoxin oxidoreductase and formaldehyde ferredoxin oxidoreductase (9, 10). Analogous tungsten enzymes have now been purified from the recently discovered organisms Thermococcus litoralis (12) which grows at temperatures up to 97 °C (12), and as yet unclassified ES-4, which grows even at 105 °C (13). Since many aldehyde-oxidizing enzymes in mesophilic organisms contain molybdenum, it appears that the catalytic functions of tungsten may be analogous to those of molybdenum in nonthermophilic organisms. Tungsten utilization may well be limited to thermophilic organisms as the only other documented cases of naturally occurring and functional tungsten enzymes are a formate dehydrogenase (14) and a carboxylic acid reductase (15–17), both from mildly thermophilic crenarchadal species. In fact, tungsten competes with molybdenum in mesophilic systems to generate nonfunctional demolybdo- or tungsten-substituted proteins (18–20).

The great majority of molybdenum-containing enzymes (the one exception being nitrogenase) utilize the metal in the form of a pterin-containing cofactor. In some of these enzymes, this cofactor consists of molybdenum ligated to a simple but unique pterin species, termed molybdopterin (see Fig. 1) (21–23), whereas in others, it contains dinucleotide forms of molybdopterin with GMP (24–27), CMP (28, 29), AMP (27), or IMP (27) appended in pyrophosphate linkage. Experiments described here were designed to address the question of whether the tungsten enzymes in aldehyde ferredoxin oxidoreductase from P. furiosus and ES-4 and the formaldehyde ferredoxin oxidoreductase from P. furiosus and T. litoralis also use molybdopterin as the organic component of their tungsten cofactors and, if so, whether the cofactors contain the simple or a dinucleotide form of the pterin. The results show that all four enzymes do contain molybdopterin. Each of the proteins yielded the well-characterized dicarboxamidomethyl derivative of molybdopterin upon denaturation with SDS in the presence of iodoacetamide (structure shown in Fig. 1) (30). Furthermore, the pterin of the tungsten cofactor of each protein was readily converted to the Form A derivative (see Fig. 1) (23). This occurs at pH 2.5 and 100 °C, conditions that are sufficient to completely hydrolyze molybdopterin dinucleotides into the pterin (Form A) and nucleotide components, which, after dephosphorylation, can be visualized in a single HPLC chromatogram. By this procedure, it was determined that no nucleotide component is present in the tungsten cofactor of these proteins. From these results, it is

* This work was supported by Grant GM00091 from the National Institutes of Health, Grant F609-88ER 13901 from the Department of Energy, and National Science Foundation Training Group Award DIR-9014281 (to the Center for Metalloenzyme Studies, University of Georgia). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 S. Mukund and M. W. W. Adams, manuscript in preparation.

2 The abbreviations used are: HPLC, high pressure liquid chromatography; camMPT, dicarboxamidomethylmolybdopterin.
concluded that the tungsten cofactor present in both the aldehyde ferredoxin oxidoreductases and the formaldehyde ferredoxin oxidoreductases has the structure shown in Fig. 1.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Enzyme Purification** — *P. furiosus* (DSM 3638) was grown using maltose as a carbon source in a medium supplemented with Na₂WO₄ (10 µM) as described previously (7). The aldehyde ferredoxin oxidoreductase was purified from 500-g batches of cells using a published procedure (8). The growth of *T. litoralis* (DSM 5473) (12) and ES-4 (13) and the purification of *T. litoralis* aldehyde ferredoxin oxidoreductase, ES-4 aldehyde ferredoxin oxidoreductase, and *P. furiosus* formaldehyde ferredoxin oxidoreductase are described elsewhere.

**Cofactor Characterization** — Preparation and isolation of camMPT were carried out as described previously (24). Enzyme samples of ~3–4 mg were diluted into 20 ml of 10 mM sodium phosphate, pH 7.0, for alkylation and denaturation. Fractions eluting from the QAE-Sephadex column with 10 mM HCl were pooled, neutralized, rotoevaporated to dryness, and taken up in 50 mM ammonium acetate, pH 6.8, for HPLC analysis. Fractions eluting with 50 mM HCl were analyzed by UV absorption spectroscopy for the presence of dicarboxamido groups, respectively. The HPLC elution profiles obtained with camMPT from all three proteins was in the range typically obtained from the molybdoenzyme sulfite oxidase.

The same enzyme preparations described above (1–1.5 mg) were used for isolation of the Form A derivative. Just prior to treatment, the samples were diluted to 1 ml with 10 mM sodium phosphate, pH 7.0, and run through a PD10 gel filtration column in the same buffer to remove the dithiothreitol and dithionite present in the storage buffer. The PD10-excluded volume, collected in 2 ml, was acidified to pH 2.5 with HCl, and 100 µl of a solution containing 1% I₂ and 2% KI was added. The sample was placed in a boiling water bath for 20 min, cooled, and adjusted to pH 8 with NaOH. The sample was centrifuged briefly, and the supernatant fraction was incubated at 4°C overnight with 25 µl of 0.5 M MgCl₂ and 1 mg of alkaline phosphatase. A portion of each sample (0.75 ml) was clarified by centrifugation through a 0.22-µm cellulose acetate membrane in a centrifuge filter unit (Costar Spin-X) and chromatographed on an HPLC column in 50 mM ammonium acetate, pH 6.8, with 7% methanol.

The instrumentation used for HPLC and UV and fluorescence spectroscopies was as previously described (24). HPLC was carried out at a flow rate of 1 ml/min. Chemicals were obtained from the following sources: alkaline phosphatase (chick intestine), Worthington; QAE-Sephadex, Sigma; SDS, Bio-Rad; and HPLC-grade methanol and ammonium acetate, Fisher. PD10 columns containing Sephadex G-25 were obtained from Pharmacia LKB Biotechnology Inc. The Form A (dephospho) standard compound (31) was provided by Professor E. C. Taylor (Princeton University).

**RESULTS**

**Isolation of camMPT from Formaldehyde Ferredoxin and Aldehyde Ferredoxin Oxidoreductases** — Fig. 2 shows the HPLC elution profile and absorption spectrum of camMPT isolated from *T. litoralis* formaldehyde ferredoxin oxidoreductase. The large peak of material with 280 nm absorbance was nonfluorescent, as expected for camMPT, and exhibited the absorption spectrum of camMPT (30). The two smaller peaks eluting at ~4 and 6 min were identified as the deamidated degradation products of camMPT with loss of two and one amido groups, respectively. The HPLC elution profiles obtained from the preparations of *P. furiosus* formaldehyde ferredoxin oxidoreductase and ES-4 aldehyde ferredoxin oxidoreductase were very similar (data not shown), and the yield of camMPT from all three proteins was in the range typically obtained from the molybdoenzyme sulfite oxidase.

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**Fig. 1.** Structures of molybdopterin, camMPT, Form A (dephospho), and tungsten cofactor of *T. litoralis* and *P. furiosus* formaldehyde ferredoxin oxidoreductases and *P. furiosus* and ES-4 aldehyde ferredoxin oxidoreductases. The pterin ring systems of molybdopterin, camMPT, and the tungsten cofactor are shown in the fully reduced tetrahydro state, but may exist in other oxidation states. Form A in the phospho or dephospho state, contains a fully oxidized pterin ring system.

**Fig. 2.** HPLC elution profile (upper) and absorption spectrum (lower) of camMPT isolated from *T. litoralis* formaldehyde ferredoxin oxidoreductase. The chromatogram was obtained with 50 mM ammonium acetate, pH 6.3, as the mobile phase. Absorbance was monitored at 280 nm. The absorbance spectrum was obtained by on-line diode array detection during chromatography. mAU, milli-absorbance units.
The yield of camMPT from *P. furiosus* aldehyde ferredoxin oxidoreductase was significantly lower, although the absorption spectrum and elution profile were identical to those of the standard compound. No UV-absorbing material was present in the fractions eluting from QAE-Sephadex with 50 mM HCl, where dicarboxamidomethylmolybdopterin guanine dinucleotide would be expected (24, 26). Nevertheless, the lower yield of camMPT from *P. furiosus* aldehyde ferredoxin oxidoreductase raised some concern that the cofactor in this enzyme might be a more complex pterin species, with only a portion that had degraded to molybdopterin amenable to alkylation and/or release by the denaturation conditions employed. A third extraction was carried out on the protein fraction retained by the Amicon membrane (which retained significant amounts of brown color). Again, the only pterin product released was camMPT. While the total yield was improved by the third extraction, it still remained lower than that obtained from the other enzymes.

Isolation of Form A from Formaldehyde Ferredoxin and Aldehyde Ferredoxin Oxidoreductases—Studies were undertaken to isolate and characterize Form A, the desulfo degradation product of molybdopterin, from the formaldehyde ferredoxin oxidoreductases and the aldehyde ferredoxin oxidoreductases and to monitor the HPLC chromatograms for evidence of any other components that may have been associated with molybdopterin in the intact enzyme-bound cofactors. The HPLC elution profiles of the dephosphorylated Form A preparations from two of the four enzymes are shown in Fig. 3. A large peak of material with 280 nm absorption eluting at ~12 min was present in both chromatograms. The material eluting in this peak had absorption and fluorescence spectra identical to those of standard Form A (dephospho), as shown in Fig. 4 (data shown for *P. furiosus* aldehyde ferredoxin oxidoreductase only). Coinjection of the Form A (dephospho) obtained from the enzyme preparations with the standard compound yielded a single peak of fluorescence eluting at 9 min in the solvent used (data for *T. litoralis* formaldehyde ferredoxin oxidoreductase shown in Fig. 5). The yield of Form A (dephospho) from *P. furiosus* aldehyde ferredoxin oxidoreductase was comparable to that obtained from the other three proteins, suggesting that the lower yield of camMPT from this enzyme could be attributed to a protein.
were clearly shown to be pterin-6-carboxylic acid on the basis of their chromatographic behavior and absorption spectra (data for *P. furiosus* aldehyde ferredoxin oxidoreductase shown in Fig. 6), indicating that the alkyl substituent was present at position 6 in the parent pterin. The absorption spectrum of pterin-7-carboxylic acid is quite different from that of the 6-carboxylate (33).

**DISCUSSION**

The identification of molybdopterin as a component of the tungsten cofactors in the aldehyde ferredoxin and formaldehyde ferredoxin oxidoreductases was not unexpected in view of the strong chemical similarities between tungsten and molybdenum. Earlier studies have demonstrated that when rats are fed a diet containing high tungsten and low molybdenum, a tungsten derivative of the molybdoenzyme sulfite oxidase is present in the liver (18, 19). Tungsten-substituted sulfite oxidase has no catalytic activity; however, the specific association of the tungsten with the normal molybdenum ligands (provided by the protein and by molybdopterin) was strongly indicated by the EPR features of the W(V) in this enzyme. The g value and saturation behavior of the W(V) signal were shifted from those of Mo(V) in the native protein as expected, while the line shape and proton hyperfine splitting of the signal were unchanged (34). Recent x-ray absorption studies of the tungsten site of *P. furiosus* aldehyde ferredoxin oxidoreductase (35) indicate that it has one thiolate and one O/N ligand in addition to the structure shown in Fig. 1, which is very similar to the molybdenum site in native sulfite oxidase. Direct evidence that the tungsten is associated with molybdopterin as a single cofactor unit in *P. furiosus* aldehyde ferredoxin oxidoreductase has recently been obtained by magnetic circular dichroism studies that show the presence of the tungsten-dithiolene.3

The presence of pterins in naturally occurring tungstoenzymes has been inferred with both formate dehydrogenase (14) and carboxylic acid reductase (17) from *Clostridium thermoaceticum*. The former protein was analyzed for the presence of molybdopterin by conversion of the pterin to the fluorescent Form A and B derivatives. Both products were demonstrated to be formed under appropriate degradation conditions. At the time of these studies, however, no attempt was made to establish whether the tungsten cofactor contained molybdopterin or a molybdopterin dinucleotide since the existence of molybdopterin dinucleotides was not recognized until more recently. The degradation conditions employed produced Forms A and B from either molybdopterin or a dinucleotide. Similarly, pterin-6-carboxylic acid was generated by permanganate oxidation of carboxylic acid reductase (17), but no further analyses were reported.

The finding that the tungsten cofactors of the aldehyde ferredoxin and formaldehyde ferredoxin oxidoreductases contain molybdopterin but not a dinucleotide is somewhat surprising. Many, but not all (29, 36), of the pterin-containing molybdoenzymes from bacterial sources use molybdopterin guanine dinucleotide, molybdopterin cytosine dinucleotide, molybdopterin adenine dinucleotide, or molybdopterin hypoxanthine dinucleotide. More specifically, several bacterial enzymes from methanogenic archaela sources that have been rigorously analyzed have been shown to contain molybdopterin guanine dinucleotide (25–27), molybdopterin adenine dinucleotide (27), and molybdopterin hypoxanthine dinucleotide (27). The specific function of the nucleotide portion of the molybdopterin dinucleotides is yet to be established. It is clear that certain molybdoenzymes, and now tungstenenzymes,

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3 R. C. Conover, S. Mukund, M. W. W. Adams, and M. K. Johnson, manuscript in preparation.
are catalytically active with unappended molybdopterin. Since the hyperthermophilic Archaea are considered the most ancient organisms known at present (1,37), it is interesting to speculate that a cofactor containing molybdopterin and tungsten but lacking a nucleotide was the ancestor of current molybdenum-containing molybdopterin diphosphates. In addition, it is important to note that the presence of the same complex, labile, and refractory molecule in enzymes from organisms ranging from the ancient hyperthermophilic Archaea to man attests to the essential nature of the molybdopterin component for the function of molybdo- and tungstoproteins in diverse life forms.

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