Characterization of a Fourth Tungsten-Containing Enzyme from the Hyperthermophilic Archaeon *Pyrococcus furiosus*

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Pyrococcus furiosus grows optimally near 100°C using peptides and carbohydrates as carbon sources, and it reduces elemental sulfur (S^0), if present, to H_2S . Tungsten (W), an element rarely used in biology, is required for optimal growth, and three different tungsten-containing enzymes have been previously purified from this organism. They all oxidize aldehydes of various types and are thought to play primary roles in the catabolism of sugars or amino acids. Here, the purification of a fourth tungsten-containing enzyme, termed WOR 4, from cell extracts of *P. furiosus* grown with S^0 is described. This was achieved by monitoring through multiple chromatography steps the W that is not associated with the three characterized tungstoenzymes. The N-terminal sequence of WOR 4 and the approximate molecular weight of its subunit determined electrophoretically (69,000) correspond to the product of an ORF (PF1961, *wor4*) present in the complete genome sequence of *P. furiosus*. WOR 4 is a homodimer and contains approximately one W, three Fe, three or four acid-labile sulfide, and one Ca atom per subunit. The visible and electron paramagnetic resonance spectra of the oxidized and reduced enzyme indicate the presence of an unusual iron-sulfur chromophore. WOR 4 does not oxidize aliphatic or aromatic aldehydes or hydroxy acids, nor does it reduce keto acids. Consistent with prior microarray data, the protein could not be purified from *P. furiosus* cells grown in the absence of S^0 , suggesting that it may have a role in S^0 metabolism.

Pyrococcus furiosus is an anaerobic archaeon that grows optimally near 100°C using peptides or sugars as the carbon and energy sources (31). The end products of its metabolism are organic acids, CO_2 , and H_2 . In addition, H_2S is produced if S^0 is added to the medium (16, 17). The growth of *P. furiosus* is dependent upon tungsten, an element rarely used in biological systems (4). Three tungsten-containing enzymes were previously purified from this organism, all of which oxidize aldehydes of various types. The enzymes are termed aldehyde ferredoxin oxidoreductase (AOR) (22, 23), glyceraldehyde-3-phosphate ferredoxin oxidoreductase (GAPOR) (24), and formaldehyde ferredoxin oxidoreductase (FOR) (26). AOR has a broad substrate specificity but is most active with aldehydes derived from amino acids (via transamination and decarboxylation) (9, 21). It is thought to play a key role in peptide fermentation by oxidizing aldehydes generated by the four types of 2-keto acid oxidoreductase present in this organism. The best substrates for FOR are C_4 to C_6 semi- and dialdehydes, and this enzyme is also thought to have a role in peptide catabolism (26). In contrast, the only known substrate for GAPOR is glyceraldehyde-3-phosphate, and it functions in the unusual glycolytic pathway that is present in *P. furiosus*, replacing the expected glyceraldehyde-3-phosphate dehydrogenase (24, 34).

The three tungstoenzymes of *P. furiosus* have subunits of similar size (~70 kDa) which have high sequence similarity ($\geq 50\%$). They constitute the so-called AOR family of tungstoenzymes, and each contains a single $[4Fe-4S]$ cluster and a

mononuclear tungsten atom that is coordinated by a bispterin cofactor (11, 19). Here we used a combination of column chromatography and tungsten analysis to purify a fourth member of this family, WOR 4, from cell extracts of *P. furiosus*. The biochemical properties of this new tungstoenzyme, which may play a role in S^0 reduction, are presented.

MATERIALS AND METHODS

Growth of the organism and protein purification. *P. furiosus* (DSM 3638) was grown in large-scale (500-liter) culture as previously described (4, 35). The medium included maltose and tryptone (each 0.5%, wt/vol) and S^0 (0.1%, wt/vol). The cultures were grown under pH-controlled conditions (pH 6.8) at 90°C until a cell-density of $\sim 4 \times 10^8$ per ml was reached. The cells were then harvested, rapidly frozen in liquid N_2 , and stored at $-80^\circ C$. WOR 4 was purified from 200 g (wet weight) under strictly anaerobic conditions at 23°C. The procedure was the same as that used to purify AOR, FOR, and GAPOR (27), up to and including the first chromatography step, except that sodium dithionite was used at 1 mM and glycerol was omitted from all buffers. WOR 4 was eluted from the first column (5.0 $\times$ 18.0 cm) of DEAE-Sepharose Fast Flow (Amersham Pharmacia Biotech Inc.) when 0.30 M NaCl in buffer A (50 mM Tris HCl, pH 8.0, containing 2 mM dithiothreitol and 1 mM dithionite) was applied. Fractions containing tungsten (350 nmol, 0.186 nmol/mg of protein) but with no significant AOR, FOR, or GAPOR activity were applied to a column (2.5 $\times$ 10 cm) of hydroxyapatite (Bio-Rad) previously equilibrated with buffer A at 4 ml/min. Adsorbed proteins were eluted with a linear gradient (2,000 ml) from 0 to 0.5 M potassium phosphate in buffer A. Tungsten-containing fractions (318 nmol, 1.25 nmol/mg of protein) eluted as 0.15 to 0.22 M potassium phosphate was applied. These were combined, diluted twofold with buffer B (50 mM bis Tris, pH 6.5, containing 2 mM dithiothreitol) and loaded on to a HiTrap Q (Amersham Pharmacia Inc.) column (three 5-ml columns connected in series). Adsorbed proteins were eluted with a linear gradient (200 ml) from 0 to 0.5 M NaCl in buffer B. Tungsten-containing fractions were eluted when 0.17 to 0.25 M NaCl was applied to the column. Those judged to be electrophoretically pure were combined, concentrated by ultrafiltration using a PM-30 membrane (Amicon, Bedford, Mass.), and stored as pellets in liquid nitrogen.

Enzyme assays. Activities of AOR (22), GAPOR (24), and FOR (26) were determined spectrophotometrically as described previously (22–24, 26, 27) under strictly anaerobic conditions under Ar in serum-stoppered cuvettes with benzyl

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FIG. 1. DEAE-Sepharose column profile during purification of WOR 4 from *P. furiosus* cell extracts. The peaks indicate either the amount of tungsten (open circles) or the specific activity of GAPOR (closed circles), FOR (closed squares), or AOR (closed triangles) in the various fractions. The diagonal line across the graph indicates the salt gradient (from 50 to 500 mM NaCl) that was applied to the column.

viologen (3 mM) as the electron acceptor. Crotonaldehyde (AOR), formaldehyde (FOR), and glyceraldehyde-3-phosphate (GAPOR) were used as substrates. Reduction of benzyl viologen was measured at 600 nm (molar absorbance, $7,800 \text{ M}^{-1} \text{ cm}^{-1}$). Results are expressed as units per milligram of protein, where 1 U equals the oxidation of 1 μmol of substrate/min/mg of protein. Hydroxy and oxo acid oxidoreductase assays were carried out at 80°C in 100 mM EPPS [*N*-(2-hydroxyethyl)piperazine-*N'*-3-propanesulfonic acid, pH 8.4] with either 2-hydroxy acids as substrates and oxidized benzyl viologen (3 mM) as the electron acceptor or 2-keto acids as substrates and reduced benzyl viologen (2 mM) as the electron donor (33). The same assays were also carried out with 100 mM potassium phosphate buffer (pH 7.0) and with NADP or NADPH as the electron mediator (2). Aldehyde/aldehyde reductase assays were carried out at 75°C in 100 mM EPPS buffer (pH 8.4) with various sugars and aldehydes as substrates (0.5 to 2 mM) and NADPH as the electron donor (13, 32).

Other methods. Colorimetric iron analysis (20), protein determinations based on a colorimetric assay (3) and corrected using quantitative amino acid analysis (24, 26), and N-terminal amino acid sequence analysis (26) were all carried out as previously described. Subunit molecular weight was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 4 to 12% bis-Tris acrylamide gel (NuPage; Invitrogen Corp.). The size of the holoenzyme was determined by Superose 6 chromatography using 50 mM Tris buffer, pH 8.0, containing 0.5 M NaCl with bovine serum albumin (molecular weight, 66,000), phosphorylase *b* (99,700), alcohol dehydrogenase (150,000), catalase (200,000), apoferritin (443,000) and thyroglobulin (669,000) as standards. Western blot analysis was performed as described previously (29) with anti-rabbit alkaline phosphatase-conjugated immunoglobulin G (Promega). A complete metal analysis (31 elements, including tungsten and iron) of protein fractions was performed using inductively coupled plasma emission spectroscopy (26). Electron paramagnetic resonance (EPR) spectra were recorded on a Bruker ER 300E spectrometer equipped with an Oxford Instruments ITC flow cryostat and interfaced to an ESP 3220 computer. Amino acid sequences were analyzed with MacVector (International Biotechnologies, Inc., New Haven, Conn.).

RESULTS

Purification of WOR 4. The presence of tungsten in fractions from chromatographic columns that did not contain AOR, FOR, or GAPOR activities was used to identify and purify WOR 4. The three previously characterized tungsten-containing enzymes can be separated from each other when a cell extract is applied to a DEAE Sepharose column (Fig. 1). GAPOR, FOR, and AOR are eluted as 100 to 160, 160 to 200,

and 190 to 260 mM NaCl, respectively, is applied to the column. The activity in each of these fractions coincides with the tungsten content. However, fractions that were eluted as 300 mM NaCl was applied also contained tungsten but had no significant AOR, FOR, or GAPOR activity (Fig. 1). They were combined and were purified by hydroxyapatite and HiTrap Q chromatography (Table 1). Fractions that had a specific tungsten content ($\sim 15 \text{ nmol/mg}$ of protein) similar to that of purified AOR, FOR, or GAPOR were obtained (5, 7, 8). From 200 g (wet weight) of frozen cells, tungsten-containing fractions that were judged to be electrophoretically homogeneous contained approximately 12 mg of protein (Table 1). For comparison, the yields of AOR, FOR, and GAPOR from the same cell mass are approximately 70, 40, and 30 mg, respectively (27). The same purification procedure could not be used to purify WOR 4 from *P. furiosus* cells grown in the absence of S^0 . In this case, tungsten could not be detected in the fractions that were eluted after AOR (300 to 390 mM NaCl) during the first DEAE Sepharose chromatography step, in contrast to the situation with S^0 -free cells (Fig. 1).

Molecular properties of WOR 4. Purified WOR 4 gave rise to a single protein band after sodium dodecyl sulfate electrophoresis (10% [wt/vol] acrylamide) that corresponded to a molecular weight of $69,000 \pm 2,000$. Analysis by gel filtration

TABLE 1. Purification of WOR 4 from *P. furiosus* cell extract

Step	Vol (ml)	Protein (mg)	Amt of tungsten (nmol)	Specific tungsten content (nmol/mg)	Purification (fold)
Crude extract	847	21,300	2,540	0.12	1.0
DEAE Sepharose	630	1,880	350	0.19	1.3
HAP ^a	310	254	318	1.25	10.5
HiTrap Q	4.5	12.3	175	14.2	119

^a HAP, hydroxyapatite chromatography.

FIG. 2. EPR spectrum of oxidized WOR 4 from *P. furiosus*. The enzyme sample (2.5 mg/ml in 50 mM Tris-HCl, pH 8.0) was oxidized with a fivefold excess of potassium hexachloroiridate ($E_m + 350$ mV). The temperature was 5.4 K, and the spectrometer settings were as follows: microwave power, 1.0 mW; gain, 5×10^4 ; time constant, 0.0409 s; sweep time, 168 s; modulation amplitude, 6.37 G, microwave frequency, 9.60 GHz.

(in the presence of 0.5 M NaCl) gave a value of $129,000 \pm 12,000$. These data suggest that the holoenzyme is a homodimer. The presence of a single subunit was confirmed by N-terminal amino acid sequence analysis of the purified protein, which gave rise to a single sequence (MFGYKGIAR). This matched exactly the translated 5' end of an ORF (PF1961 or *wor4*) in the genome sequence of *P. furiosus* (25) that would encode a 623-residue protein with a molecular weight of 69,363, in good agreement with the estimated mass of WOR 4. The translated amino acid sequence of *wor4* shows at least 49% amino acid sequence similarity with the sequences of AOR, FOR, and GAPOR of *P. furiosus* (26), showing that these proteins are part of the same (AOR) family. However, antibodies that had been generated to each of the latter three tungstoenzymes did not show any significant cross-reactivity with purified WOR 4 (data not shown).

Purified WOR 4 contained 0.71 ± 0.05 g-atoms of tungsten, 2.7 ± 0.4 g-atoms of iron, 0.80 ± 0.01 g-atoms of calcium, and 0.33 ± 0.01 g-atoms of zinc atoms per subunit (69,393 g of protein) when analyzed by plasma emission spectroscopy and 2.7 ± 0.4 iron atoms/subunit and 3.5 ± 0.5 S²⁻ atoms/subunit by colorimetric analysis (each value is the average of at least two determinations each using two different enzyme preparations). No other metals were present in significant amounts (>0.1 g-atom/subunit). WOR 4 as purified gave a broad visible absorption peak near 420 nm, the intensity of which increased considerably upon addition of potassium ferricyanide. The visible absorption decreased by about 50% upon addition of sodium dithionite, a characteristic of an iron-sulfur chromophore. Surprisingly, dithionite-reduced WOR 4 (80 μ M protein) did not display any significant EPR in either the $g \sim 2$ or $g \sim 4$ region of the spectrum (recorded at 4 K and 10 mW; data not shown). On the other hand, when the protein was oxidized with hexachloroiridate, it gave rise to two overlapping axial-type signals at temperatures up to 70 K (Fig. 2). Together these represented approximately 0.4 spin/mol.

Catalytic properties of WOR 4. All three of the characterized tungstoenzymes of *P. furiosus* catalyze the oxidation of aldehydes of one sort or another (35). In light of this and the similarity of the sequence of WOR 4 to those of these other enzymes, the ability of WOR 4 to oxidize aldehydes and catalyze related reactions was investigated. However, no activity was detected at 80°C with formaldehyde, glyceraldehyde, propionaldehyde, crotonaldehyde, glutaraldehyde, isovaleraldehyde, glyceraldehyde-3-phosphate, salicylaldehyde, benzaldehyde, or phenylpropionaldehyde as a substrate (0.5 to 10 mM concentration range) and with either benzyl or methyl viologen (3 mM) as the electron acceptor. In addition, no activity was detected in the reverse reaction, acid reduction, using formate, acetate, or glutarate as substrates (0.5 to 5.0 mM) with dithionite-reduced methyl viologen as the electron donor. WOR 4 was also examined for its ability to reversibly oxidize 2-hydroxycarboxylic acids. This stemmed from the similarity of the N-terminal amino acid sequence of the enzyme 2-hydroxy acid:viologen oxidoreductase from *Proteus vulgaris* (33) and *Clostridium tyrobutyricum* (2) with those of the AOR family, including WOR 4. However, WOR 4 did not oxidize lactate, 2-hydroxybutyrate, 2-hydroxycaproate, or 1-ethyl-2-hydroxycaproate, nor did it reduce the 2-keto acids pyruvate, 2-ketobutyrate, 2-ketocaproate, and 2-ketovalerate, using benzyl viologen or NAD(P)(H) as the electron mediator. In addition, WOR 4 did not exhibit aldehyde or aldose reductase-type activity. It did not reduce aldehydes such as glyceraldehyde, propionaldehyde, benzaldehyde, glucose, and galactose (1 to 10 mM) with NAD(P)H or dithionite-reduced benzyl viologen as the electron donor. Furthermore, none of these reductase-type activities were detectable in cell extracts of *P. furiosus*.

DISCUSSION

WOR 4 is the fourth tungstoenzyme to be purified from cell extracts of *P. furiosus*. The subunit size of this homodimeric protein (69.3 kDa) is comparable to those of its relatives, homodimeric AOR (66.3 kDa), homotetrameric FOR (69.0 kDa) and monomeric GAPOR (73.0 kDa), and WOR 4 shows 58% (37%), 57% (36%), and 49% (25%) amino acid sequence similarity (identity), respectively, with these enzymes. Structural studies show that each subunit of AOR and FOR contains one [4Fe-4S] cluster coordinated by four cysteinyl residues that is situated about 10 Å from a mononuclear tungsten site (6, 10). While WOR 4 also seems to contain a single W atom per subunit, the measured Fe and sulfide contents are consistent with either a [4Fe-4S] or a [3Fe-4S] center. In fact, sequence comparisons with AOR and FOR indicate that the corresponding FeS cluster in WOR 4 would be coordinated by only three cysteinyl residues with a noncoordinating glycine (residue 288) replacing the expected fourth cysteine (26). This would favor a 3Fe rather than a 4Fe cluster (12), in which case reduced WOR 4 (containing a [3Fe-4S]⁰ center) would be EPR silent, as was observed. However, the EPR signal exhibited by oxidized WOR 4 is not that expected from an oxidized [3Fe-4S]⁺ cluster (12); rather, the g values, line shape, and temperature dependence of the EPRs (Fig. 2) are characteristic of an oxidized high-potential iron-sulfur protein (or Hipip)-type [4Fe-4S]³⁺ center existing in two EPR-distinguishable confor-

mations (7). We therefore conclude that WOR 4 contains an unusual type of FeS cluster, and data from other spectroscopic techniques are obviously required to ascertain its nature. The absence of any EPRs from the dithionite-reduced enzyme that might originate from W(V) is presumably due to the inability of dithionite to reduce the low potential W(VI) site, as observed with some other tungstoenzymes (11). Like FOR (but not AOR or GAPOR), WOR 4 also contains approximately one Ca atom/subunit, although crystallographic analyses indicate that the Ca atom in FOR has a structural rather than a catalytic role (10). Thus, unfortunately, the metal and cluster content of WOR 4, while showing some unexpected features, provide no insight into the catalytic properties of this protein.

WOR 4 was unable to oxidize any of the aliphatic or aromatic aldehydes, 2-hydroxycarboxylic acids, or sugar aldehydes that were tested using viologen dyes or nicotinamide nucleotides as mediators. Nevertheless, analysis of the *P. furiosus* genome sequence (25) suggests that WOR 4 may have a role in aldehyde conversions. Unlike the genes encoding AOR, FOR, and GAPOR, which are nonlinked and are separated from neighboring ORFs by at least 40 nucleotides, the start codon of *wor4* is directly adjacent to the stop codon of a putative ORF (PF1960) that is annotated as an aldose/aldehyde reductase (25). This suggests that WOR 4 (PF1961) might use or produce aldehydes and/or aldoses associated with PF1960. However, this putative reductase is a member of a ubiquitous family of enzymes that catalyze the NADPH-dependent reduction of a diverse range of aldehydes and/or sugars to alcohols (1, 5, 32, 36) and identifying the reaction catalyzed by the *P. furiosus* protein is not possible from sequence information alone. For example, the protein encoded by this ORF shows ~70% sequence similarity to the 6'-deoxychalcone synthase of soybean (36), the aldo/keto reductase isoform 1 of humans (5), the polyketide reductase of *Glycerhria echinata*, (1), and the aldehyde reductase of *Acinetobacter* sp. strain M-1 (32).

Insight into the function of WOR 4 is also not evident from an examination of other microbial genomes. Homologs of WOR 4 are present in other hyperthermophilic archaea, such as the anaerobes *Pyrococcus horikoshii* (14) and *Archaeoglobus fulgidus* (18) and the microaerophile *Pyrobaculum aerophilum* (8), as well as in the moderately thermophilic aerobes *Thermoplasma volcanium* (15) and *Thermoplasma acidophilum* (28). These appear to be true homologs, as their sequences are more similar to that of WOR 4 (approximately 40% sequence similarity) than they are to those of AOR, FOR, and GAPOR (26). A property common to all of these organisms is not obvious, other than the fact that they are all archaea. It may be significant that the *P. furiosus* cells used to purify WOR 4 were grown in the presence of S^0 and that the protein could not be purified by the same methods from cells grown in the absence of S^0 . In accord with this result, DNA microarray analyses show that expression of *wor4* is almost threefold higher in maltose-grown *P. furiosus* when S^0 is present (30). However, WOR 4 did not exhibit polysulfide reductase activity using reduced ferredoxin as the electron donor (using extracts of S^0 -grown cells as a positive control; data not shown). Thus, why WOR 4 appears to be S^0 responsive is not clear at present, and determining the precise function of this enzyme will not be an easy task, especially in the absence of genetic tools for *P. furiosus*. Nevertheless, the availability of the pure WOR 4 protein

should enable such studies to be complemented with immunological analyses, and these are in progress.

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