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Molybdenum incorporation in tungsten aldehyde oxidoreductase enzymes from *Pyrococcus furiosus*†

Running title: *Pyrococcus furiosus* molybdenum incorporation

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Abstract

The hyperthermophilic archaeon *Pyrococcus furiosus* expresses five aldehyde oxidoreductase (AOR) enzymes, all containing a tungsto–bispterin cofactor. The growth of this organism is fully dependent on the presence of tungsten in the growth medium. Previous studies have suggested that molybdenum is not incorporated in the active site of these enzymes. Application of the radioisotope ^{99}Mo in *Metal Isotope native RadioAutography in Gel Electrophoresis* (MIRAGE) technology to *P. furiosus* shows that molybdenum can in fact be incorporated in all five AOR enzymes. Mo(V) signals characteristic for molybdopterin were observed in formaldehyde oxidoreductase (FOR) in EPR–monitored redox titrations. Our finding that the aldehyde oxidation activity of FOR and WOR5 (W–containing oxidoreductase 5) correlates only with the residual tungsten content suggests that the Mo containing AORs are most likely inactive. An observed W/Mo antagonism is indicative of tungstate–dependent negative feedback of the expression of the tungstate/molybdate ABC transporter. An intracellular selection mechanism for tungstate and molybdate processing has to be present, since tungsten was found to be preferentially incorporated into the AORs even under conditions with comparable intracellular concentrations of tungstate and molybdate. Under the employed growth conditions of starch as the main carbon source in a rich medium, no tungsten and/or molybdenum–associated proteins are detected in *P. furiosus* other than the high–affinity transporter, the proteins of the metallopterin insertion machinery, and the five W–AORs.

Introduction

Molybdenum (Mo) and tungsten (W) are transition metals with comparable chemical properties, playing an essential role in biological systems. They are present in similar cofactors of enzymes, which consist of one or two tricyclic pterin moieties, usually called molybdopterin or, alternatively, metallopterin (MPT). The cofactors that contain Mo are called Moco and those that contain W are called Wco (or recently Tuco [22]). Mo and W are widely distributed in biology; however, they are not universal bioelements. Mo is present in many forms of life, e.g., as an essential trace element in humans as well as in most bacteria of which the genome has been sequenced, whereas W–containing enzymes have been indentified in prokaryotes, mainly in archaea, such as the hyperthermophilic archaeon *Pyrococcus furiosus* [9], but also in some mesophilic bacteria such as the human food borne pathogen *Campylobacter jejuni* [29, 30]. The best characterized W enzymes are from *P. furiosus,* a marine anaerobic, fermentative archaeon, which grows optimally at 100 °C. The hyperthermophilic archaeon *P. furiosus* was originally isolated from shallow marine volcanic sediment off the coast of the island Vulcano in Italy. Interestingly, *P. furiosus* is fully dependent on W for growth and can discriminate between molybdate and tungstate from the environment, having a preference for tungstate over molybdate. It can use peptides or polycarbohydrates as a carbon source, producing mainly acetate [9] with the production of molecular hydrogen from protons as terminal electron acceptor [26]. *P. furiosus* expresses five different aldehyde oxidoreductases (AORs) each containing a tungsto–bispterin cofactor, namely, formaldehyde: ferredoxin oxidoreductase (FOR) [25], aldehyde: ferredoxin oxidoreductase (AOR) [19], glyceraldehyde–3–phosphate: ferredoxin oxidoreductase (GAPOR) [17], tungsten–containing oxidoreductase 4 (WOR4) [24], and tungsten–containing oxidoreductase 5 (WOR5) [3]. In addition to the five genes encoding AOR proteins, the *P. furiosus* genome encodes two hypothetical proteins, PF1242 and PF1521, that share homology with formate dehydrogenases from *Desulfovibrio gigas*, which might contain either Mo or W. So far, no physiological function has been identified for Mo in *P. furiosus*. However, a recent study has shown that a tungstate binding protein, WtpA, part of the tungstate ABC transporter (WtpABC) from *P. furiosus*, binds both oxoanions, molybdate and tungstate, albeit with different affinities [4]. Therefore, it is expected that *P. furiosus* is able to take up molybdate from its environment with the WtpABC transporter.

Whereas previous attempts by Adams and co–workers to incorporate Mo or vanadium in the active site of these AOR enzymes have been unsuccessful [18], we here report the incorporation of Mo in all five AOR enzymes. A recently developed method, Metal Isotope native RadioAutography in Gel Electrophoresis (MIRAGE) [27], was used in combination with the short–lived radioisotope ^{99}Mo to visualize Mo containing proteins in *P. furiosus*. The quantity of the metal inside the separate spots after native–native two–dimensional

polyacrylamide gel electrophoresis (2D–PAGE) was determined using autoradiography. This method was previously used with the short–lived radioisotope ^{187}W to identify the W metalloproteome of *P. furiosus* under different growth conditions [28], and was found to compare well with transcriptomic data [32]. In the present paper we describe the incorporation of Mo in enzymes of the *P. furiosus* AOR family using the radioisotope ^{99}Mo.

Material and methods

Growth of the organism – (i) *Growth of the organism for MIRAGE – P. furiosus* (DSM 3638) cultures were grown anaerobically at 90 °C using potato starch as carbon source, as previously described [9]. The growth was performed in 100–mL serum bottles filled with 50 mL growth medium. Three batchwise growth conditions were used for comparison: (A) normal–W growth, radioactive Mo, 10 μM $NaWO_4$ in addition to 400 μM ^{99}Mo–spiked MoO_4^{2-} (here referred to as $^{99}MoO_4^{2-}$); (B) normal–W growth, radioactive W, 10 μM ^{187}W–spiked WO_4^{2-} (here referred to as $^{187}WO_4^{2-}$) in addition to 400 μM MoO_4^{2-} and (C) low–W growth, radioactive Mo, 20 nM $NaWO_4$, in addition to 400 μM $^{99}MoO_4^{2-}$.
(ii) *Growth of the organism for protein purification – P. furiosus* (DSM 3638) was grown in an 8–liter fermentor at 90 °C, under anaerobic conditions with starch as carbon source as previously described [2] . For cells grown under low–tungstate and high–molybdate conditions, a preculture without added tungstate was required. To minimize traces of tungstate present in the cells of the inoculum, which was derived from regular tungstate–grown cells, 10 subsequent precultures were made in medium containing 20 nM sodium tungstate and 10 μM sodium molybdate. The final culture, which contained effectively 20 nM tungstate, was used for the inoculation of the 8–liter fermentor. Completely omitting tungstate from the medium resulted in no growth. After inoculation of the 8–liter fermentor and 18 h running in batch mode, the culture was switched to continuous mode with a dilution rate of 0.3 h^{-1} [21] resulting in a wet weight of approximately 2 g/L. FOR and WOR5 were purified from 175 g cells (wet weight) under anaerobic conditions at 23 °C as previously described [3, 25].

Preparation of the radioisotopes solution – A quartz vial containing 4 mg of highly enriched $^{98}MoO_3$ powder was sealed and irradiated for 10 h at the Reactor Institute Delft (Delft, The Netherlands) using a thermal neutron flux of 5.0×10^{16} $m^{-2}s^{-1}$. After irradiation, the vial was allowed to cool to room temperature for 5 h to ease handling and to decrease the radiation from short–lived radioisotopes in the quartz vial and the aluminium irradiation tray. The vial content that consisted of $^{99}MoO_3$ was dissolved in 150 μL of 10% NaOH and neutralized with 36% HCl. The obtained solution, which contained $^{99}MoO_4^{2-}$ and NaCl, was added to the growth medium. A small amount of the radioisotope–containing solution was measured in a germanium detector for gamma–ray spectroscopy in order to calculate the

amount of ^{99}Mo (half–life $t_{1/2}$ = 66 h) and the purity of this radioisotope. The $^{187}WO_4^{2-}$ solution was prepared as previously described [28].

Protein sample preparation for MIRAGE – After cultivation, the *P. furiosus* cells were spun down using a Jouan CR 4.11 centrifuge at 3,500 rpm at 5 °C for 1 h and were resuspended in 1 mL 50 mM Tris–HCl pH 8 containing 0.1 mg/L DNase and 0.1 mg/L RNase. The cells were broken by osmotic shock. A homogenized native soluble protein extract was obtained after vortexing with a home–made bead beater at 2,500 rpm at 5 °C for 30 min, and broken cells were spun down in an Eppendorf centrifuge at 14,000 rpm at 5 °C for 1 h. The soluble extract was subsequently concentrated using an ultrafiltration device (Microcon YM3; Millipore) with 3–kDa cutoff. The protein concentration was determined with a bicinchoninic acid assay kit (Uptima–Interchim) according to the manufacturer's instructions. A small amount of the protein sample was measured using gamma–ray spectroscopy with a high purity germanium detector (GEM20P–Ortec), in order to check for any contaminating (i.e., not ^{99}Mo) radionuclides.

Membrane protein sample preparation for MIRAGE – As described above, the cells were spun down and resuspended in 10 mL 50 mM Tris–HCl, pH 8, containing 0.1 mg/L DNase and 0.1 mg/L RNase. Cell debris and unbroken cells were removed by centrifugation at 3,000 rpm at 5 °C for 30 min, and supernatant was decanted and centrifuged at 100,000×g at 5 °C for 2 h using a Beckman L8–70 ultracentrifuge. The membrane pellet was washed two times with 10 mL of 1 M NaCl by resuspending the pellet using a bead beater at 2,500 rpm at 5 °C for 1 h. A new membrane pellet was obtained by centrifugation at 100,000×g at 5 °C for 2 h. The washed membrane pellet was resuspended and homogenized in a buffer containing 1% 3[(3–cholamidopropyl)dimethylammonio]–propanesulfonic acid (CHAPS), 750 mM aminocaproic acid in 50 mM Tris–HCl pH 7, and this constituted the solubilized membrane protein fraction. The protein concentration was determined using the bicinchoninic acid assay. The radioactivity retained in the membranes after each washing step was measured using a gamma counter (Wizard 3 automatic gamma counter, Wallac, Perkin–Elmer).

MIRAGE – *Metal Isotope native RadioAutography in Gel Electrophoresis* was used to determine the intracellular Mo– and W–containing proteins from *P. furiosus*. This sensitive metalloproteomic technology was recently developed [27], and involves four steps: (I) labelling of target proteins with a radioisotope; (II) separation of intact holoproteins using native isoelectric focusing (IEF), followed by blue native PAGE (BN-PAGE) in the second dimension; (III) spot visualization and quantification using autoradiography; and (IV) protein identification by tandem mass spectrometry (MS/MS) after in–gel trypsin digestion.

The first and second dimensions of the native–native polyacrylamide gel electrophoresis (2D–PAGE) were carried out as previously described [27]. Briefly, native IEF was performed using a Multiphor II flatbed electrophoresis system (GE Healthcare) at 15 °C. Linear immobilized pH gradient (IPG) gel strips, pH 3–11 or pH 4–7, 24 cm, were used for the separation of the native proteins based on their isoelectric point (pI). In both cases, the protein sample was applied at pH 4.2 by cup–loading (in the case of membrane solubilized extract this was applied by incubating the IPG strips overnight with a reswelling solution containing the protein). IEF of 0.1–0.7 mg protein sample was performed at 3,500 V for 12 h continuously after an initial 1 min at 500 V, ramping up to 3,500 V in 1.5 h (gradient). After the first–dimension separation, the IPG strips were equilibrated as previously described [27]. The second dimension, BN 2D–PAGE was performed on the same electrophoresis system, but at 5 °C on a precast homogenous 12.5% acrylamide separation gel (ExcelGel 2D, GE Healthcare). The BN–PAGE was run in two steps: 120 V for 1.5 h at 20 mA and 3 W, followed by 600 V for 2.5 h at 30 mA and 20 W. Autoradiograms were taken off the IPG strips before and after the second dimension and of the second dimension BN–PAGE gel. Amounts of ^{99}Mo and ^{187}W were quantified using a multipurpose phosphor screen (12.5 cm × 25.2 cm), a Cyclone phosphorimager, and OptiQuant software (all from Perkin–Elmer). Phosphor screens were scanned with a resolution of 600 dpi. The image resolution was reduced to a final resolution of 60 dpi by integration over 10 by 10 pixels using Matlab 7.4. Subregions were selected for three–dimensional (3D) visualization using Tecplot 360 2008. The phosphor screens were calibrated as previously described [27, 28], the only difference being that ^{99}Mo was used instead of ^{64}Cu or ^{187}W. A calibration curve from the ^{99}Mo standard solution with a known specific activity was used to quantify the amount of Mo in spots produced in the IEF and native 2D–PAGE experiments (see Figure S1 in the supplemental material). IEF, 2D–PAGE gels, and calibration spots were read with the same phosphor screen to enable a quantitative correction for the decay of ^{99}Mo. Following the alignment of the autoradiogram and the original gel, the separated spots were excised. After radioactivity decay for more than 10 times the radioisotope half–life, the excised protein spots were *in–gel* digested with trypsin and analyzed by tandem MS as described previously [27, 33]. Using the calibration curve of ^{99}Mo, digital light units (DLU) were converted to Mo amounts (pmol). Absolute expression levels of the Mo (holo)proteins for the different growth conditions, as based on the ^{99}Mo quantification, were corrected for protein losses as determined from the recovery of ^{99}Mo after the different electrophoresis steps.

AOR activity assay – Enzyme activities were routinely assayed at 60 °C, under anaerobic conditions, with aldehyde substrate and with 1 mM methylviologen as the electron acceptor in 50 mM 4–(2–hydroxyethyl)–1–piperazinepropanesulfonic acid (EPPS) buffer, pH 8.4. As described earlier, the following substrates characteristic for particular AOR enzymes were

use: crotonaldehyde (for AOR) [19], formaldehyde (for FOR) [25], glyceraldehyde–3–phosphate (for GAPOR) [17].

Spectroscopy – Redox titrations were performed at room temperature under argon in 50 mM Hepes, pH 7.5 containing 4 mg/mL (58 μM of monomer) FOR. The following redox dyes were added to a final concentration of 50 μM: N,N,N',N'–tetramethyl–p–phenylenediamine, 2,6–dichlorophenol indophenol, phenazine ethosulfate, methylene blue, resorufine, indigodisulfonate, 2–hydroxy–1,4–naphtaquinone, anthraquinone–2–sulfonate, phenosafranin, safranin O, neutral red, benzylviologen, and methylviologen. Samples were first reductively titrated with sodium dithionite followed by oxidative titration with potassium ferricyanide. After equilibration at the desired potential a 0.2–mL sample was anaerobically transferred to an Electron Paramagnetic Resonance (EPR) tube and immediately frozen in liquid nitrogen. Potentials were measured with a platinum electrode versus an Ag/AgCl reference electrode. All reported redox potentials are with respect to the normal hydrogen electrode (NHE). A substrate/product (formaldehyde/formate) titration was performed at room temperature under argon in 50 mM Hepes, pH 7.5, using the same concentration of FOR (58 μM of monomer). The protein was incubated with formaldehyde and formate at the following ratios (calculated reduction potential): 1:99 (–472 mV), 1:9 (–502 mV), 1:1 (–532 mV), 9:1 (–562 mV), and 99:1 –592 mV). These are the low–potential points in the redox titration that could not be reached with dithionite as reductant. These potentials were required to EPR detection of low–potential W and Mo species in FOR. After equilibration at the desired potential, a 0.2–mL sample was anaerobically transferred to an EPR tube and immediately frozen in liquid nitrogen. X–band EPR spectra were recorded on a Bruker ER 200D spectrometer, using facilities and data handling as detailed elsewhere [20].

Determination of Mo and W in seawater from the natural habitat of *P. furiosus* – Electroanalytical Mo and W determination from seawater of the coast of the island Vulcano, Italy, sampled just above to the sea floor at circa 2 m close to the site where *P. furiosus* was originally reported to be collected [9]. Electrochemical measurements were performed with a digitally controlled PSTAT10 potentiostat using GPES 4.8 software (Eco–chemie, The Netherlands) equipped with a hanging mercury drop working electrode (HMDE 663 VA stand, Metrohm Switzerland), a glassy carbon counter electrode, and a double–junction Ag/AgCl (3 M KCl) reference electrode (Metrohm). Oxine, 3–methoxy–4–hydroxymandelic acid, sodium molybdate, and sodium chlorate were obtained from Sigma. Mercury (analytical reagent [AR] grade) and sulfuric acid were obtained from Merck. Mo and W were determined by catalytic–adsorptive stripping voltammetry (AdSV) according to the method in reference 14. Aliquots of 50 μl seawater sample or 5 μl 10 μM sodium molybdate or sodium tungstate standard solution were added to a 20–mL blank solution containing 10 mM H_2SO_4, 0.5 mM oxine,

0.05 mM 3–methoxy–4–hydroxymandelic acid, and 50 mM $NaClO_3$ in an electrochemical cell thermostatted at 30 °C. For the AdSV the following conditions were used: pulse height, 25 mV; step potential, 5 mV; deposition potential, 0.10 V; deposition time, 120 s; stirring speed during deposition 1,000 rpm; scan range, 0.10 to –1.0 V. The mercury drop surface was approximately 0.52 mm^2. Prior to measurement the solution was purged with high–purity argon for 300 s. The Mo peak was observed at –0.227 V and the W peak at –0.676 V versus Ag/AgCl. Three replicates of the seawater sample, three molybdate standard additions and three tungstate standard additions were measured.

Determination of intracellular free and protein–bound Mo and W of *P. furiosus* – The intracellular free and protein–bound molybdenum and tungsten concentration of *P. furiosus* were determined using catalytic–adsorptive stripping voltametry as previously described [28]. Three 100–mL serum bottles, filled with 50 mL growth medium, were used for overnight (14 h) cultivation at 95 °C of *P. furiosus*. The cells (140 mg) were spun down as described above and the pellet was washed with 15% NaCl to remove extracellular tungstate and molybdate, without disrupting the cells. The cells were introduced into an anaerobic glove box and resuspended in 1.5 mL 50 mM Tris–HCl, pH 8.0, containing 0.1 mg/L DNase and 0.1 mg/L RNase. Cell debris and unbroken cells were removed by centrifugation at 14,000 rpm at 5 °C for 1 h using an Eppendorf centrifuge, and the supernatant was concentrated using a Microcon filter (Millipore) with 3–kDa cutoff. The protein–containing samples were digested as previously described with 10% (wt/vol) perchloric acid [14]. Precipitated proteins were removed by centrifugation at 14,000 rpm for 10 minutes. The Mo and W content was measured in the filtrate, the concentrate, and the original cell extract. The intracellular volume was calculated using the ratio of 4.5 µl volume/mg of protein for *P. furiosus* as reported previously [16].

Results

Method optimization – For each neutron irradiation, 4 mg of enriched $^{98}MoO_3$ was used to obtain 25 ± 5 MBq of $^{99}MoO_3$. After correction for the traces of nonradioactive Mo in the growth medium a specific activity of 6.25×10^9 Bq/g was obtained. An average half–life of 65.8 hours was found for ^{99}Mo, which is in a good agreement with the reported half–life of 66 ± 0.15 h [13]. Gamma–ray spectroscopy (using a germanium detector) showed that the irradiated sample contains 99 ± 0.5% ^{99}Mo, with 1% in total of traces of different radioactive metals. These traces may encompass a small amount of W which was converted to ^{187}W ($t_{1/2}$ = 23.8 h) upon irradiation. Gamma–ray spectroscopy of the desalted and concentrated protein sample (cell extract) obtained after growing the cells, showed a ^{99}Mo purity of 99.9 ± 0.1%.

The decay of the radioactivity in the separated protein spots after gel electrophoresis followed that of ^{99}Mo, thereby excluding significant incorporation of ^{187}W contamination.

Molybdenum is incorporated into all five tungsten enzymes by *P. furiosus* – ^{99}Mo–MIRAGE was applied for *P. furiosus* cells for different growth conditions. Figure 1 shows the autoradiograms of ^{99}Mo–MIRAGE of *P. furiosus* soluble–protein extract in each of the growth conditions used. The MS/MS results typically show several overlapping proteins for each excised spot. Using the *P. furiosus* genome annotation, the entities relevant to the tungsten–molybdenum metalloproteome became evident. The complete lists of protein identification data for all analyzed spots are compiled in Tables 1 and 2 (Figure 1 showing the respective spot positions on the gels). Detailed MS results are assembled in Tables S1 and S2 in the supplemental material.

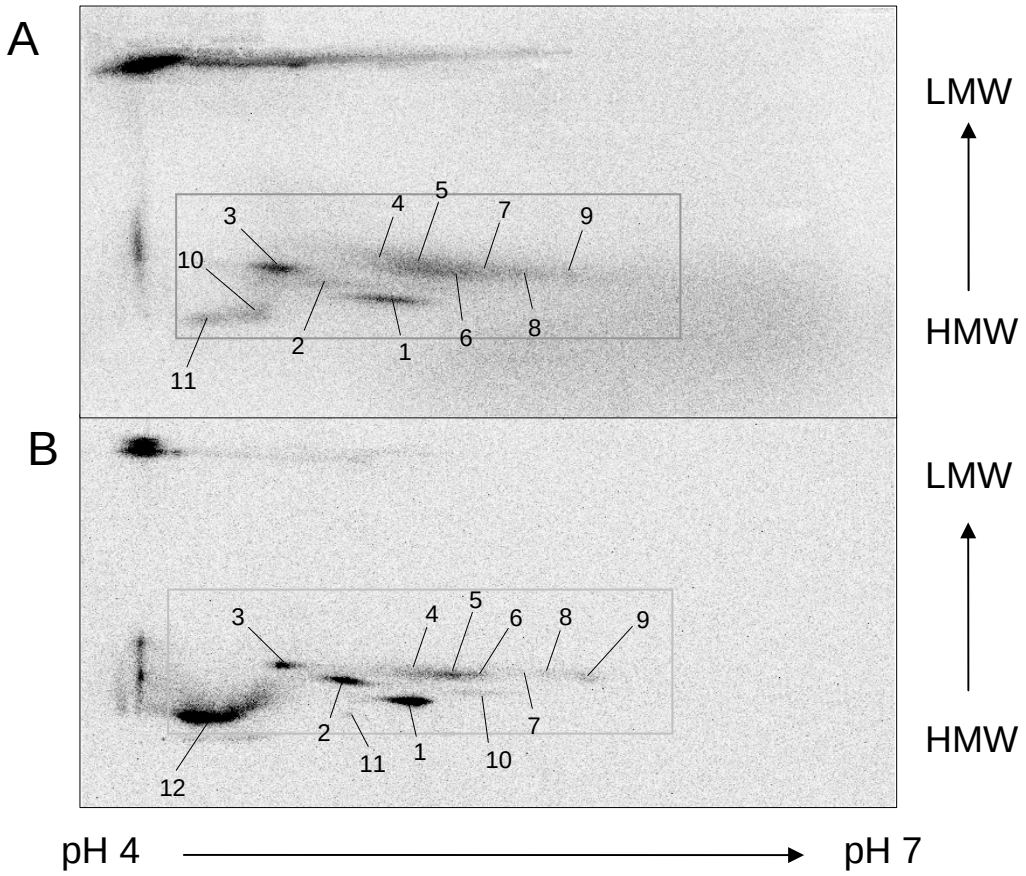

Figure 1 Autoradiogram of native 2D–PAGE of soluble protein extract of *P. furiosus*. Extracts were obtained from cells grown in **A** 10 µM WO_4^{2-} and 400 µM $^{99}MoO_4^{2-}$ (550 µg protein) and in **B** 20 nM WO_4^{2-} and 400 µM $^{99}MoO_4^{2-}$ (375 µg protein). LMW, low molecular weight; HMW, high molecular weight.

Table 1 Protein identification by liquid chromatography–MS/MS after native–native 2D–PAGE separation of *P. furiosus* soluble protein extract obtained from growth under 10 μM sodium tungstate and 400 μM sodium molybdate $^{99}MoO_4^{2-}$.

Spot	Locus[a]	Protein	Mass	Number of unique peptides	Mascot[b] Protein Score
1	PF1203	FOR	69,072	19	804
2	PF0464	GAPOR	74,089	2	82
	PF1480	WOR5	65,025	1	40
3	PF0346	AOR	66,931	22	1094
	PF0464	GAPOR	74,089	3	103
4	PF1961	WOR4	69,610	12	636
	PF1203	FOR	69,072	6	278
	PF0464	GAPOR	74,089	7	236
	PF0542	MoeA1	43,164	1	74
5	PF1961	WOR4	69,610	24	1,454
	PF1203	FOR	69,072	18	1,014
	PF0464	GAPOR	74,089	8	345
	PF0542	MoeA1	43,164	1	76
6	PF1961	WOR4	69,610	19	971
	PF1203	FOR	69,072	10	420
	PF0464	GAPOR	74,089	6	218
	PF0542	MoeA1	43,164	2	97
7	PF1961	WOR4	69,610	7	451
	PF0464	GAPOR	74,089	7	266
	PF1203	FOR	69,072	4	164
8	PF0464	GAPOR	74,089	8	312
	PF1961	WOR4	69,610	4	221
	PF1203	FOR	69,072	6	209
9	PF0464	GAPOR	74,089	8	325
	PF1961	WOR4	69,610	1	60
10[c]	–	–	–	0	–
11[c]	–	–	–	0	–

[a]Locus on the *P. furiosus* genome [23].
[b]Mascot identity threshold $p < 0.05$. Spot numbers correspond to numbered positions in Figure 1A.
[c]Spots 10 and 11 do not contain any Mo or W related proteins. These spots are attributed to inorganic polymolybdate species formed during the preparation of the ^{99}Mo containing solution.

Table 2 Protein identification by liquid chromatography–MS/MS after native–native 2D–PAGE separation of *P. furiosus* soluble protein extract obtained from growth under 20 nM sodium tungstate and 400 μM sodium molybdate $^{99}MoO_4^{2-}$.

Spot	Locus[a]	Protein	Mass	Number of unique peptides	Mascot[b] protein score
1	PF1203	FOR	69,072	27	1,379
2	PF1480	WOR5	65,025	19	977
	PF1783	MoeA2	43,858	6	278
3	PF0346	AOR	66,931	28	1671
4	PF1203	FOR	69,072	20	690
	PF1961	WOR4	69,610	18	662
	PF0464	GAPOR	74,089	4	116
5	PF1961	WOR4	69,610	27	1,108
	PF1203	FOR	69,072	19	686
	PF0464	GAPOR	74,089	7	178
	PF0543	MoaD[c]	10,276	1	20
6	PF1961	WOR4	69,610	12	527
	PF1203	FOR	69,072	15	474
	PF0464	GAPOR	74,089	4	129
7	PF0464	GAPOR	74,089	8	263
8	PF0464	GAPOR	74,089	12	427
	PF1961	WOR4	69,610	3	159
	PF1480	WOR5	65,025	1	44
9	PF0464	GAPOR	74,089	14	391
	PF1961	WOR4	69,610	3	132
10	PF0542	MoeA1	43,164	3	36
11	–	–	–	0	–
12[d]	–	–	–	0	–

[a]Locus on the *P. furiosus* genome [23].
[b]Mascot identity threshold $p < 0.05$. Spot numbers correspond to numbered positions in Figure 1B.
[c]MoaD is not expected to contain Mo or W, but is involved in the Mo– and W–cofactor biosynthesis.
[d]Spot 12 does not contain any Mo or W related proteins. This spot is attributed to inorganic polymolybdate species formed during the preparation of the ^{99}Mo containing solution.

We have also applied ^{187}W–MIRAGE on *P. furiosus*. The cells were grown under normal ^{187}W growth, 10 μM $^{187}WO_4^{2-}$ in addition to non–radioactive 400 μM MoO_4^{2-}. With this experiment we could directly compare the incorporation of ^{99}Mo versus ^{187}W in the AOR enzymes under the same cultivation conditions. The autoradiograms are presented in Figure S4 in the supplemental material. From all proteins expressed under different growth conditions the metal radioisotopes were quantified in each of the excised spots (Table 3).

Table 3 Metal quantification from the native–native 2D PAGE gels of soluble protein extract (normalized to 375 µg protein). Calculated amounts (pmol) of Mo and W in *P. furiosus* AOR–family enzymes based on metal radioisotopes ^{99}Mo and ^{187}W.

	Normal–W[b] High Mo	Normal–W High Mo	Low–W High Mo
	Mo	**W**	**Mo**
FOR	0.14 ± 0.01[a]	30.8 ± 1.1	0.90 ± 0.06
AOR	0.13 ± 0.04	9.2 ± 0.8	0.20 ± 0.05
GAPOR	0.08 ± 0.01	7.6 ± 0.5	0.14 ± 0.04
WOR4	0.15 ± 0.01	7.2 ± 0.2	0.22 ± 0.07
WOR5	0.06 ± 0.01	8.0 ± 0.5	0.39 ± 0.01

[a]Error value was defined as standard deviation for the quantification of spots from two independent gels for each condition.
[b]Conditions: normal–W: 10 µM WO_4^{2-}, high Mo: 400 µM MoO_4^{2-}, low–W: 20 nM WO_4^{2-}.

The 3D images of the ^{99}Mo–MIRAGE and ^{187}W–MIRAGE autoradiograms are presented in Figure 2. These show the position of AOR enzymes on the gel. Their quantifications based on the amount of ^{187}W or ^{99}Mo are presented in Table 3. Only for some observable features in the acidic part of the autoradiograms could no Mo or W containing enzymes be identified by MS/MS proteins analysis. Therefore, a ^{99}Mo–MIRAGE control experiment was performed on the $^{99}MoO_4^{2-}$ solution only (i.e., without protein extract), and the same features were found at around pH 4 (see Figure S2 in the supplemental material). We attribute these to inorganic polymolybdate and polytungstate species formed during the preparation of the ^{99}Mo or ^{187}W [28] solution (Figures 1 and 2; see also Figure S4). Indeed, the same features can also be seen in the autoradiograms of the solubilized membrane proteins (Figure 3B; see also Figure S3).

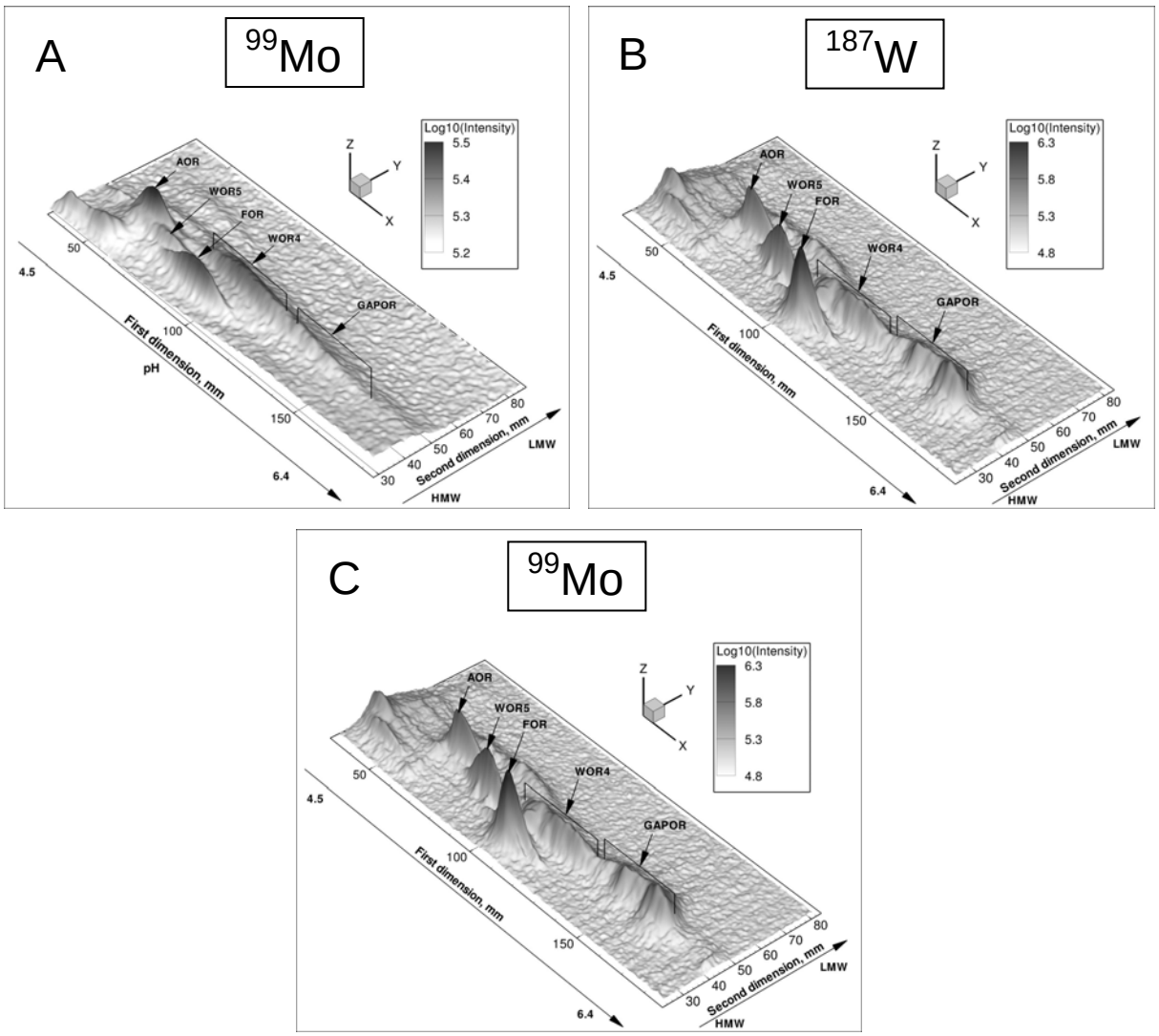

Figure 2 A 3D image of ^{99}Mo–MIRAGE of *P. furiosus* soluble protein extract (550 µg protein) obtained from cells grown in 10 µM WO_4^{2-} and 400 µM $^{99}MoO_4^{2-}$, **B** 3D image of ^{187}W–MIRAGE of *P. furiosus* soluble protein extract (650 µg protein) obtained from cells grown in 10 µM $^{187}WO_4^{2-}$ and 400 µM MoO_4^{2-}, **C** 3D image of ^{99}Mo–MIRAGE of *P. furiosus* soluble protein extract (375 µg protein) obtained from cells grown in 20 nM WO_4^{2-} and 400 µM $^{99}MoO_4^{2-}$.

Mo was incorporated by all five AOR enzymes to a level of 0.5 to 2% compared to the W incorporation. The Mo incorporation under conditions with low–tungstate increased strongly in FOR and WOR5, and only a minor increase was observed for the other three enzymes.

The intracellular free and protein–bound molybdenum and tungsten concentrations of *P. furiosus* cultivated under the three growth conditions (as used for the MIRAGE experiments) are presented in Figure 4. Under normal growth conditions (10 µM WO_4^{2-} and no added MoO_4^{2-}), there was virtually no free and no protein bound Mo. Under these conditions approximately 20% of the intracellular W is not bound to proteins, which is

consistent with previously published results [28]. Under growth conditions with normal–W and high Mo (10 µM WO_4^{2-} and 400 µM MoO_4^{2-}), a significant intracellular Mo concentration was found, with only a small fraction associated to proteins. When *P. furiosus* was cultivated under low–W and high–Mo conditions (20 nM WO_4^{2-} and 400 µM MoO_4^{2-}), the intracellular W concentration was lower than the intracellular Mo concentration. However the W was exclusively bound to protein, while the Mo was predominantly "free" (not bound to protein). As a result, the intracellular protein bound W concentration was still higher than in the intracellular protein–bound Mo concentration. The protein–bound Mo concentration, however, was significantly higher in cells grown under low–W than under normal–W conditions.

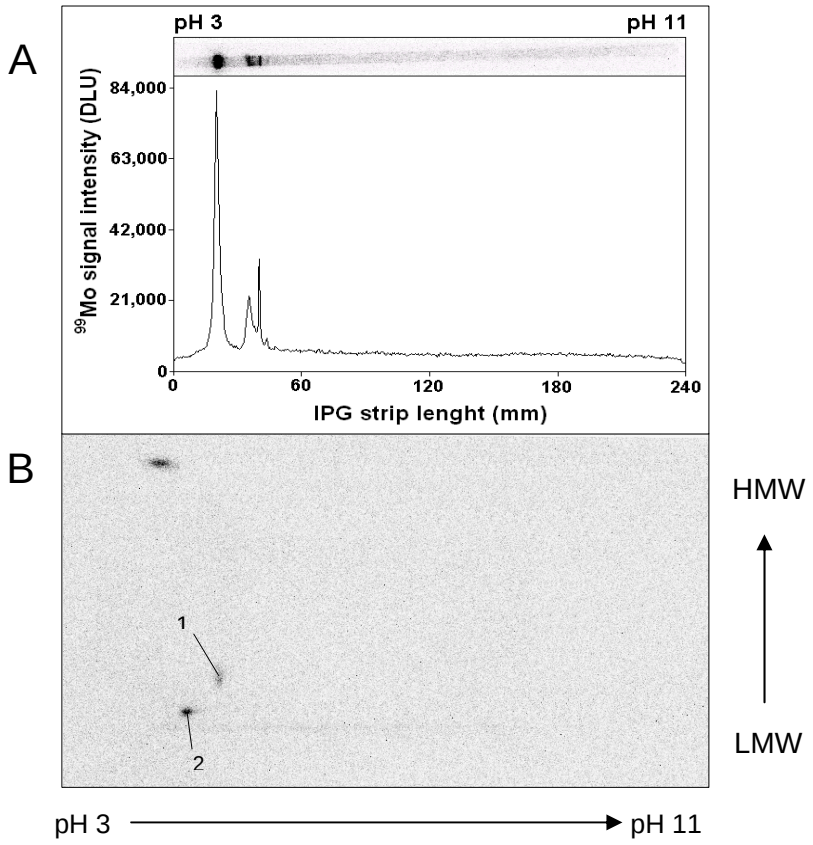

Figure 3 Autoradiogram of IEF **A** and native–native 2D–PAGE **B** of solubilized membrane protein extract of *P. furiosus*. Membrane extract (180 µg protein) was obtained from cells grown in 20 nM WO_4^{2-} and 400 µM $^{99}MoO_4^{2}$–. Spot 2 does not contain any Mo– or W–related proteins. This spot is attributed to inorganic polymolybdate species formed during the preparation of the ^{99}Mo containing solution. LMW, low molecular weight; HMW, high molecular weight.

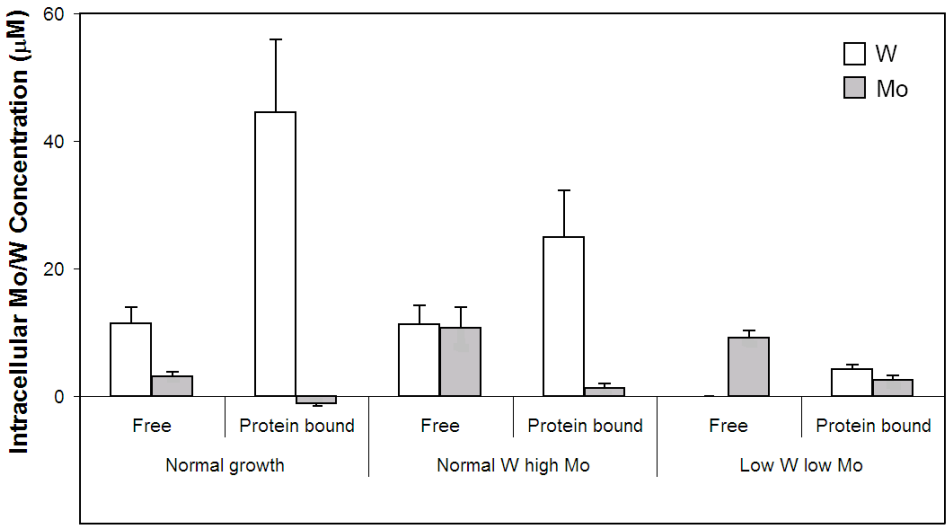

Figure 4 Intracellular Mo and W concentrations (µM) of *P. furiosus* cells grown under different conditions: Normal–W (10 µM WO_4^{2-} and no added MoO_4^{2-}); Normal–W and high Mo (10 µM WO_4^{2-} and 400 µM MoO_4^{2-}) and Low–W and high Mo (20 nM WO_4^{2-} and 400 µM MoO_4^{2-}). The negative value for protein bound Mo after normal growth is a consequence of the subtraction of the Mo value of the filtered cell extract (3 kDa cutoff) and the original cell extract and uncertainties in these values.

Intracellular distribution of molybdenum – The MIRAGE results of *P. furiosus* under high–molybdate/high–tungstate and high–molybdate/low–tungstate conditions indicate that FOR and WOR5 incorporate circa 6.5 times more Mo upon a decrease of the tungstate by 3 orders of magnitude while for the other enzymes (AOR, GAPOR, and WOR4) the Mo content increases only 1.5– to 2–fold (Figure 2 and Table 3). We remark that MIRAGE does not give information on the protein level *per se*, only on the absolute metal quantity. We therefore do not draw conclusions on protein expression levels. However, it can be inferred from our data that there is a difference in the metal content of the AOR enzymes as a response to the tungstate/molybdate concentration.

The amount of W associated with GAPOR is relatively high in *P. furiosus* cultivated under high–tungstate and high–molybdate conditions, as shown by ^{187}W–MIRAGE (Figure 2). The GAPOR associated molybdate is very low under these conditions.

The *P. furiosus* genome encodes two paralogs of MoeA, the enzyme responsible for the metal incorporation into the molybdopterin cofactor: MoeA1 and MoeA2 [5]. The reason why *P. furiosus*, and indeed most tungsten using–organisms, has two versions of this enzyme remains enigmatic. In the light of the present results, it is tempting to suggest that the two paralogs are targeted at different enzymes of the AOR family. Here we have found, for the first time that both paralogs are expressed in this organism, as both MoeA1 and MoeA2 were found during the MIRAGE experiments. MoeA2 was found to colocalize with WOR5,

whereas MoeA1 was found in spots overlapping with WOR4 (with minor amounts of FOR and GAPOR) (Table 1 and 2). Under low tungstate, MoeA1 was also detected in a Mo containing spot with no apparent Mo or W enzymes.

Evidence for regulation of tungsten uptake – After cell disruption, removal of the soluble protein extract and cell debris, and two washing steps with high salt concentration, a relatively high content of ^{99}Mo was still detected in the membrane protein containing pellet. The membrane proteins from *P. furiosus* were solubilized and analyzed with ^{99}Mo–MIRAGE (Figure 3). No significant difference in the autoradiogram patterns was found for solubilized membrane protein extracts from *P. furiosus* grown on 20 nM WO_4^{2-} and 400 µM $^{99}MoO_4^{2-}$ versus 10 µM WO_4^{2-} and 400 µM $^{99}MoO_4^{2-}$ (Figure 3B; see also Figure S3 in the supplemental material). An additional ^{99}Mo spot (Figure 3B) was identified after cultivation with low–W as compared to normal–W. This spot was found to contain the protein WtpA based on the detection of eight unique peptides (see MS details in Tables S3 and S4 in the supplemental material). WtpA is a periplasmic binding protein as part of an ABC transporter system selective for tungstate and molybdate, and its expression in *P. furiosus* has not been shown before [4]. The fact that the WtpA spot was only present after growth on low concentrations of tungstate (20 nM) suggests that, by analogy to the well characterized ModABC molybdate transport system from *Escherichia coli*, the expression of WtpABC may be negatively regulated by the intracellular tungstate concentration [11]. The *P. furiosus* genome, however, does not encode a ModE (molybdenum–responsive regulatory protein in *E. coli*) homolog; therefore, the candidate transcriptional regulator remains elusive.

Molybdenum–containing AOR enzymes are inactive – The cell extracts obtained from cells grown on excess of molybdate and from those grown on an excess of tungstate were both examined for their total aldehyde oxidation activity using three characteristic substrates (formaldehyde, crotonaldehyde, and glyceraldehyde–3–phosphate) in order to estimate the levels of expression of the three most abundant aldehyde oxidoreductases in *P. furiosus*: AOR, FOR, and GAPOR (Figure 5). A 100–fold–higher formaldehyde oxidation activity was found in the tungstate–supplemented–cells compared with the molybdate–supplemented–cells.

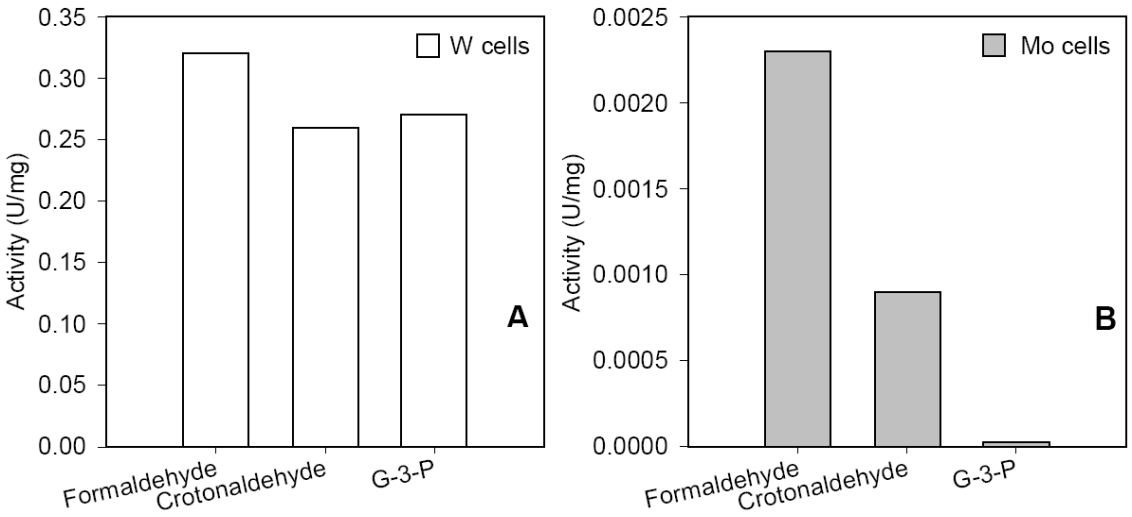

Figure 5 Specific activity of formaldehyde, crotonaldehyde and glyceraldehyde–3–phosphate (G–3–P) oxidation assayed at 60 °C, under anaerobic conditions, and 1 mM methyl viologen as electron acceptor in 50 mM EPPS, pH 8.4, in cell extracts of *P. furiosus* cells grown in medium supplemented with tungstate **A** or molybdate **B**. The medium with normal tungstate contained 10 μM W and 90 nM Mo, the medium with high–molybdate contained 20 nM W and 10 μM Mo.

In Table 4 the activity and metal content of isolated FOR and WOR5, produced under different W and Mo concentrations, are compared. The ratio of the activity over the metal content (Figure 6) shows that the activity of the AORs correlated well with the residual W content and not with the Mo content. Therefore, we conclude that Mo incorporation into the AOR family enzymes results in inactive enzymes (at least, under the assay conditions used here).

Table 4 Purification table for FOR and WOR5 purified from cells grown on tungstate or molybdate[a]

	CFE	Purified FOR			Purified WOR5		
Medium	U/mg	U/mg	Mo/subunit	W/subunit	U/mg	Mo/subunit	W/subunit
W	0.32	27	0.0064	0.48	6.5	0.011	0.13
Mo	0.0032	1.25	0.057	0.02	0.95	0.23	0.017

[a]The formaldehyde oxidation activity (U/mg) was assayed at 60 °C, under anaerobic conditions with 50 mM formaldehyde as substrate and 1 mM methyl viologen as electron acceptor in 50 mM EPPS, pH 8.4.

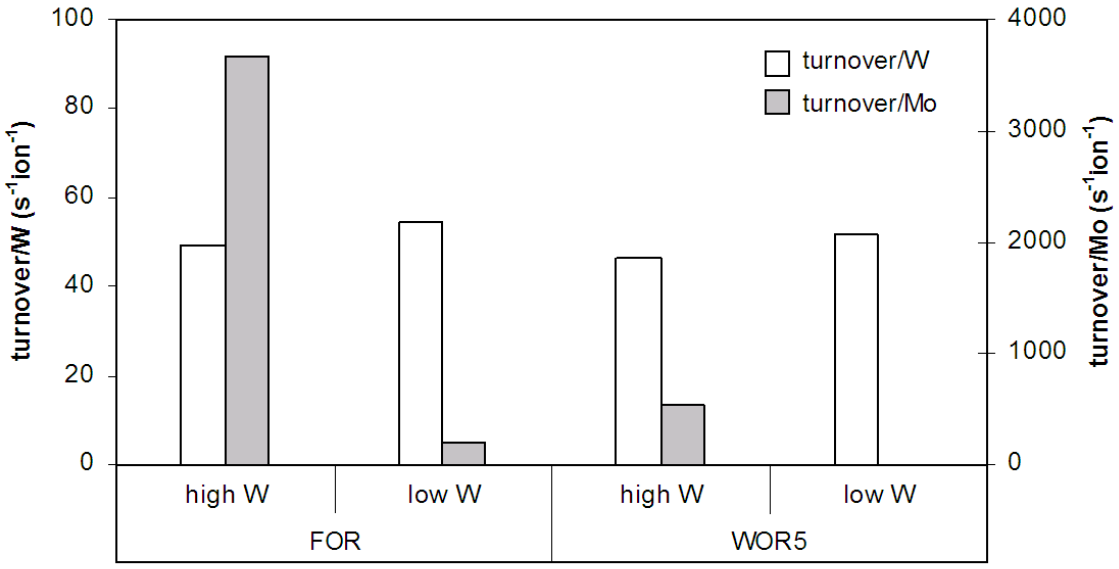

Figure 6 Correlation between AOR activity and W/Mo metal content. Data used for the calculation of the turnover per metal ion are derived from Table 4.

EPR Spectroscopy FOR – Purified W–FOR (i.e., from growth on high–tungstate) and Mo–FOR (i.e., from growth on high–molybdate) were comparatively subjected to EPR spectroscopy. Figure 7 shows spectra from dithionite–reduced Mo–FOR (trace a), dithionite–reduced W–FOR (trace b), their difference spectrum (trace c), and a simulation of the difference (trace d). The spectrum in trace b was previously characterized as 'low–potential' W(V) with g_{zyx} values 1.969, 1.901, and 1.847 [7]. This signal is also observed in Mo–FOR; however, there is also a new signal, which is retained in the difference spectrum and simulated with *g* values significantly higher (g_{zyx} = 2.04, 1.969, and 1.925) than those of the tungsten signal, as would be expected in case of a Mo(V)/W(V) substitution of the metallopterin. Although the traces in Figure 7 are each averages of nine spectra, the afforded signal–to–noise ratio is insufficient to allow inclusion of Mo hyperfine structure in the simulation. Previously, a signal from midpotential W(V), with g_{zyx} = 1.977, 1.946, and 1.926, was found in a redox titration at intermediate potentials [7], and we now find an equivalent midpotential Mo(V) signal in a redox titration of the Mo–FOR (trace e), again with significantly higher *g* values (g_{zyx} = 2.015, 1.976, and 1.950). The Mo(IV/V) reduction potentials are not measurably different form those of the W(IV/V) couples as estimated from formaldehyde/formate titrations of the low–potential component ($E_m \approx -0.45$ to –0.5 V) and from mediated redox titration of the midpotential component ($E_m \approx -0.25$ V) [7]. The S=3/2 and S=1/2 signals from the $[4Fe–4S]^{1+}$ cubane and its $E_m \approx -0.33$V are essentially identical in W–FOR and Mo–FOR (not shown).

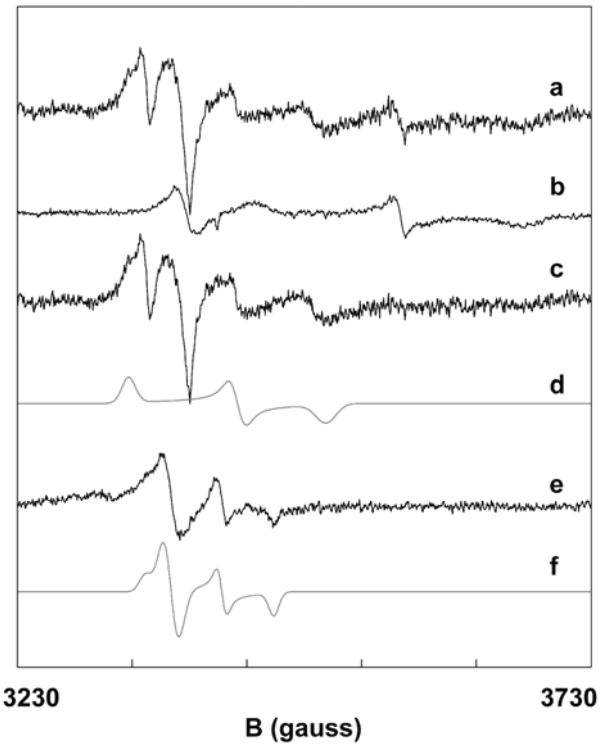

Figure 7 EPR spectroscopy of W(V) and Mo(V) in *P. furiosus* formaldehyde oxidoreductase. The proteins were circa 10 $mg \cdot mL^{-1}$ in 50 mM Hepes, pH 7.5. The difference spectrum (trace c) of dithionite–reduced Mo–FOR (trace a) and W–FOR (trace b) is simulated (trace d) as a single low–potential Mo(V) component with g_{zyx} values of 2.024, 1.969, and 1.925 and line widths (Gaussian standard deviations) (W_{zyx}) of 7, 7, and 10 G. Mo–FOR poised at a potential of –0.2 V (trace e) is simulated (trace f) as a single midpotential Mo(V) component with g_{zyx} values 2.015, 1.976, and 1.950 and W_{zyx} values of 6, 4, and 5 G plus a minor radical signal with a g_{iso} value of 2.002. EPR conditions: microwave frequency, 9.43 GHz; microwave power, 50 mW; modulation frequency, 100 kHz; modulation amplitude, 6.3 G; temperature, 50 K.

Mo and W concentrations in the natural habitat of *P. furiosus* – The seawater sample that was taken off the coast of the island Vulcano (Italy), the natural habitat of *P. furiosus*, was found to contain 0.18 ± 0.03 μM Mo and circa 0.03 μM W. The W level was close to the detection limit for samples containing high NaCl levels. The order–of–magnitude–higher concentration of Mo over W provides a clear explanation for the need for a selective tungstate uptake system (WtpABC) in *P. furiosus*, which allows the preferential uptake of W in the presence of excess Mo.

Discussion

Previous attempts to substitute tungsten with molybdenum or with vanadium in the AOR enzymes of *P. furiosus* were unsuccessful. Adams and coworkers grew *P. furiosus* in excess of molybdate or vanadate, and they purified AOR enzymes by monitoring aldehyde oxidation activity and subsequently determined their metal content. No incorporation of Mo or V was detected in the AORs within the detection limits of the plasma emission spectroscopy used for the metal analysis, and the authors concluded that Mo is not incorporated in the active sites of AOR enzymes [18].

By applying the more sensitive MIRAGE (metal isotope native radioautography in–gel electrophoresis) in combination with ^{99}Mo on *P. furiosus* cells, we reveal a different picture.

The method can measure, depending on the half–life of metal radioisotopes and their specific activities, subpicomole amounts of metal in a protein sample, and, therefore, typically is more sensitive than alternative metal analysis techniques, including inductively coupled plasma MS (ICP–MS). With ^{99}Mo–MIRAGE applied to *P. furiosus*, all five AOR enzymes were found to contain/incorporate substoichiometric amounts of Mo. Besides the AOR family of enzymes, no other Mo–containing proteins were found. The amount of incorporated Mo was found to be W concentration dependent: more Mo was incorporated in WOR5 and FOR when cells were grown under conditions of low tungstate. No GAPOR activity could be detected, although this enzyme is required for glycolysis. It is known that *P. furiosus* has two enzymes, glyceraldehyde–3–phosphate dehydrogenase (GAPDH) and phosphoglycerate kinase (PGK), that together can take over the role of GAPOR [31].

EPR spectra confirmed the incorporation of Mo into the pterin cofactor of FOR. In the Mo-containing sample, previously unobserved signals were detected at redox potentials similar to those known for W-FOR [7]; therefore, they were named low- and midpotential forms of Mo.

The expression of the *E. coli* molybdate transport system, ModABC, as well as part of the Moco biosynthesis machinery is regulated by the transcriptional regulator ModE [11]. The dimeric ModE protein binds tungstate or molybdate with the same affinity (K_D = 0.8 μM) [1] and is, therefore, not able to discriminate between tungstate and molybdate. There is no apparent *modE* homolog in the genome of *P. furiosus*, the expression product of which could regulate tungstate/molybdate uptake as in *E. coli*. The W binding protein WtpA from the tungstate ABC transporter (WtpABC) from *P. furiosus* has previously been shown to bind both oxoanions with a high affinity [4]. This result would exclude the transporter system as a selective barrier in favor of tungstate over molybdate when present in an approximate 1:1,000 ratio as in typical natural seawater. Therefore, *P. furiosus* should be able to take up molybdate from the media with the WtpABC transporter. Alternatively, molybdate at 400 μM may be taken up by low affinity transporters such as sulphate transporters, as has been described in bacteria [12]. The *P. furiosus* genome encodes several putative sulphate transporters (PF1519, PF1520, and PF1522 and PF1748, PF1749, and PF1750) that may serve as low–affinity molybdate transporters. This was a major motivation to repeat the Mo substitution experiments in *P. furiosus* AOR proteins. The seawater from the natural habitat of *P. furiosus* was found to contain only an order of magnitude higher [Mo] than [W]. The WtpABC would be sufficient to take up W from the *P. furiosus* natural environment. In our work we have proven that, even at much higher Mo/W ratio the enzymes incorporate preferentially W, showing there is an intracellular selection mechanism. The MIRAGE results were confirmed by independent determination of the intracellular "free" and protein–bound Mo and W concentrations. In *P. furiosus* cultivated under conditions with low–W and high–Mo, significant intracellular Mo was found; however the protein–bound W level was still higher

than the protein–bound Mo level. This clearly demonstrates the intracellular preference of *P. furiosus* for W over Mo.

Comparing ^{187}W–MIRAGE with ^{99}Mo–MIRAGE for the same growth condition (10 µM WO_4^{2-} in addition to 400 µM MoO_4^{2-}) shows that all AOR enzymes prefer W incorporation over Mo. W cannot be fully replaced by Mo in AOR enzymes in *P. furiosus*, but for all AOR enzymes it is possible to incorporate substoichiometric amounts of Mo. FOR and WOR5 incorporated Mo up to seven times more when cells were grown in low–W than under normal–W conditions. The other W–containing enzymes (AOR, GAPOR, and WOR4) have a comparable Mo incorporation in the two growth conditions used, with a slightly higher incorporation of Mo in the presence of low–W during growth. However, these Mo–containing AORs do not contribute to the observed aldehyde oxidation activity. Despite the fact that there is enough intracellular molybdate (see Figure S5 in supplemental material), there is only substoichiometric incorporation of molybdate into the AOR enzymes. Apparently there is also intracellularly a strong preference for tungstate over molybdate, suggesting the existence of a selective mechanism for W incorporation in the pterin cofactor.

In *E. coli* selective metal incorporation is thought to rely on the metal–dependent hydrolysis of adenylylated MPT coupled to the metal insertion activity of MoeA [15]. In *P. furiosus* there are two MoeA paralogs: MoeA1 (PF0542) and MoeA2 (PF1783). Compared to each other, the *P. furiosus* proteins share 40% identity and 58% similarity and compared to *E. coli* MoeA the numbers are 35% identity and 53% similarity for MoeA1 and 32% identity and 50% similarity for MoeA2.

It is tempting to speculate that the expression of these proteins forms the basis of metal (Mo) selectivity. Both proteins were found in ^{99}Mo–MIRAGE experiments to be colocalized with AORs: MoeA1 colocalized with WOR4 and MoeA2 with WOR5. We have applied recombinant MoeA1 and MoeA2 to the native 2D PAGE procedure. However, these proteins do not run to clearly different positions than the AOR–family enzymes, so we cannot exclude the possibility that the colocalization of MoeA1 and MoeA2 with the WOR proteins is coincidentally, rather than reflecting a stable protein complex. Interestingly, MoeA1 and MoeA2 were not found in previous ^{187}W–MIRAGE experiments, suggesting that the colocalization of MoeA1 and MoeA2 with the AORs after ^{99}Mo–MIRAGE may represent stalling of the metalloenzyme maturation in the presence of Mo.

WtpA has been shown to bind tungstate with higher affinity than molybdate. It has a significant off–rate *in vitro* and loses all its tungstate within a few hours in an environment without tungstate [4]. This may well have been the reason why it has not been possible to visualize this protein previously with ^{187}W–MIRAGE, when it was expressed under normal tungstate concentrations [28]. By applying ^{99}Mo–MIRAGE on solubilized membrane protein extracts under low–tungstate growth conditions and excess of radioactive molybdate, we here, for the first time, successfully isolate and identify WtpA as Mo–containing protein. Our

detection of WtpA in the membrane protein fraction suggests that WtpA is bound to the rest of the transporter system WtpBC, although these proteins were not identified (presumably due to the well–known difficulties in the analysis of membrane proteins, which often lack sufficient soluble tryptic peptides for unambiguous identifications). Alternatively, immature WtpA which contains a targeting signal peptide may still be anchored to the membrane. Without signal peptide, WtpA is a soluble protein [6]. We believe that the Mo is trapped in the WtpABC complex (or just WtpAB) and, therefore, cannot be released in the absence of ATP. The mechanism of tungstate transport by *Archaeoglobus fulgidus* WtpABC has been proposed to involve conformational changes of the two transmembrane WtpB subunits that can be either inward facing when WtpC is nucleotide free, or outward facing when WtpC has ATP bound [10]. Perhaps in the absence of ATP, a WtpABC complex can be trapped in a conformation that strongly retains tungstate or molybdate, with the inward facing WtpB dimer covered by bound WtpA. Under this condition WtpA may not be able to release its metal ion.

Genomic analysis indicated that, besides the W containing enzymes of the AOR family, there are three genes with significant sequence homology to genes of known W– or Mo–containing proteins. These genes encode PF1242 and PF1521, two putative formate dehydrogenase (FDH) [23] –like proteins, and PF2025, a putative homolog to the CuMoS cluster–containing orange protein (ORP) which has been purified and characterized from *Desulfovibrio gigas* [8]. PF2025 and *D. gigas* ORP share 32% sequence identity and 46% similarity. Despite the fact that transcription of the two putative FDH–encoding genes has been shown in DNA microarray experiments [32], no evidence for the expression as a soluble or membrane–bound Mo– or W–containing protein has been found here or in ^{187}W–MIRAGE experiments [28]. It is possible that the expression of these FDH–like proteins and of the ORP is below our detection limit of 50 fmol under the employed growth conditions.

In conclusion, by applying ^{99}Mo–MIRAGE on *P. furiosus* we have found that all five AOR enzymes incorporate substoichiometric amounts of Mo, resulting in an inactive form of the enzymes. EPR signals indicative for Mo(V) in molybdopterin were observed in Mo–containing formaldehyde oxidoreductase (FOR) in redox titrations. Besides AOR enzymes, no other protein was found to contain Mo. The periplasmic binding protein WtpA as part of an ABC transporter system selective for tungstate and molybdate could be found only after growth on low concentrations of tungstate (20 nM) and high concentration molybdate (400 μM) which suggests that the expression of WtpABC may be negatively regulated by the intracellular tungstate (and not molybdate) concentration.

Acknoledgements

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