Identification of YdhV as the First Molybdoenzyme Binding a bis-Mo-MPT Cofactor in Escherichia coli

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CONTENTS

Supplementary Tables and Figures

TABLES

- Table S1: Element contents in YdhV from TXRF.
- Table S2: W EXAFS simulation parameters.
- Table S3: YdhV metal quantification and spin concentrations from EPR spectroscopy.
- Table S4: Comparison of spin Hamiltonian parameters for selected molybdoenzymes.

Table S5: Fe EXAFS simulation parameters of YdhV.

FIGURES

- Figure S1: Sequence alignment of YdhV and BamBC (A) and Crystal structure of BamBC (B).
- Figure S2: Elemental analysis with TXRF.
- Figure S3: X-band EPR spectra of apo-YdhV.
- Figure S4: EPR spectral simulation of the Mo(V) signal of ferricyanide-oxidized YdhV at 150 K.
- Figure S5: Power-dependent relaxation behavior of paramagnetic species in YdhV.
- Figure S6: Wide-sweep X-Band EPR spectra of YdhV at low temperature.

Figure S7: Fe XAS of YdhV.

proporation	ele	ement per prot	ein
preparation	Р	Fe	Br
YdhV +Cl	1.7(5)	2.3(9)	0.1(1)
YdhV +Br	1.5(2)	2.9(5)	0.5(1)

Table S1: Element	contents in	YdhV from	TXRF. ^a
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^aData stem from two protein samples for each preparation and 3 repetitions per sample (standard deviation in parenthesis) and were determined relative to a gallium standard added to the protein solutions. Ni, Cu, and Zn contents were ≤ 0.4 metals per protein on average. From a series of YdhV samples to which increasing concentrations of a phosphorus standard were added (Supporting Figure S2), a P per protein ratio of 1.8(2) was determined.

	W=0,-0H,-0	W=S	W-S _{MPT,cys}	W-Cl ^b ,W ^c	$R_{\rm F}$
		N [per Mo] / R [Å] / $2\sigma^2 x 10^3$ [Å ²]		[%]
YdhV 10 µM W	0.5 [#] / 1.72 / 2 [*] 0.4 [#] / 1.81 / 2 [*]	0.5# /2.24 / 2*	4.0* / 2.40 / 5 0.6# / 2.53 / 2*	^b 0.1 / 2.79 / 2	19.2
YdhV 1 mM W	1.6 / 1.74 / 4 [§] 2.3 / 1.93 / 4 [§] 4.6 / 4.00 / 12 [§]	-	-	°4.4 / 3.45 / 12 ^{\$*}	21.4
Na ₂ WO ₄ pH 4.0	2.0 / 1.74 / 8 [§] 1.9 / 1.91 / 8 [§] 6.5 / 3.57 / 2 [§]	-	-	°0.8 / 3.51 / 2	28.1
Na ₂ WO ₄ pH 7.1	3.0 / 1.77 / 5§ 1.0 / 2.10 / 5§	-	-	-	23.0
Na ₂ WO ₄ pH 10.6	3.9 / 1.78 / 2	_	-	_	16.4

Table S2: W EXAFS simulation parameters.^a

^aN, coordination number; R, interatomic distance; $2\sigma^2$, Debye-Waller factor; R_F, fit error sum (deviation between Fourier back-transform in a 1.0-3.5 Å reduced distance window and simulation curve). Fit restraints: *fixed parameter, #N-values coupled to yield a sum of 2, ^{§,§}coupled to yield the same value. Parameters correspond to EXAFS simulations in Figure 6. ^bParameters correspond to a W-Cl distance, ^cparameters correspond to a W-W distance.

Table S3: YdhV metal quantification and spin concentrations from EPR.

The concentrations of YdhV were determined by A_{280nm} absorbance, while the concentrations of Mo, W, and Fe were determined by ICP-OES. The spin concentrations were determined through use of the Matlab toolbox 'spincounting' from the obtained spectra and associated tune picture of the resonator. The reported spin/YdhV relates the amount of spin to YdhV present. Quantitated spin, unless noted otherwise, were detected from 80 K spectra measured with 4 mW microwave power, 5 G modulation amplitude, and 100 kHz modulation frequency.

YdhV; Treatment	YdhV (uM)	Mo (µM)	W (µM)	Fe (µM)	Spin (uM)	Spin/YdhV
apo-YdhV $(+MoO_4^{2-})^{1,2}$	1094	1328	N/D	2100		
as-iso dithionite-reduced ferrricyanide- oxidized					104 147 72	0.095 0.134 0.066
apo-YdhV $(-MoO_4^{2-})^1$	662	5	N/D	927		
as-isolated dithionite-reduced (12 K) ³					2 ⁷ 60 57	0.003 0.091 0.086
ferrricyanide- oxidized					47	0.006
YdhV-Mo ^{2,5}	202	380	N/D	288		
as-isolated dithionite-reduced ferrricyanide- oxidized (150 K) ⁴					5 10 17 41	0.040 0.080 0.084 0.203
YdhV-W ^{5,6}	221	N/D	977	N/D		
as-isolated dithionite-reduced ferrricyanide- oxidized					1^7 28 4^7	0.005 0.126 0.018

N/D not detected.

¹YdhV variant was purified and expressed aerobically. The bound Fe-S cluster was highly similar between aerobic and anaerobic expressions and purifications, respectively.

²YdhV was expressed in the presence of 1 mM MoO₄²⁻.

³Acquisition parameters at 8 μ W microwave power, 10 G modulation amplitude, and 100 kHz modulation frequency.

⁴Acquisition parameters at 4 mW microwave power, 5 G modulation amplitude, and 100 kHz modulation frequency.

⁵YdhV variant with optimal loading of bis-Mo-MPT, as detected by EPR spectroscopy. YdhV was expressed in the $\Delta mobA/\Delta mocA$ deletion strain and was expressed and purified anaerobically.

 6 YdhV was expressed in the presence of 10 μM WO4 $^{2-}$.

⁷It should be noted that the reported quantity of spin is at the limit of detection, therefore representing a negligible spin quantity.

Table S4: **Comparison of spin Hamiltonian parameters for selected molybdoenzymes.** Table denotes signal type for the specified organism and enzyme, and associated *g*-tensor parameters reported, either by simulation or where applicable, by assignment. Where available, the associated ^{95,97}Mo hyperfine values, in addition to assigned Euler angles, are provided. Where applicable, associated nuclear quadrupole values and hyperfine values to nuclei other than Mo are excluded. Reported YdhV simulation parameters are depicted from Figure S4.

Organism	Signal Type	a-values	σ	$\sigma_1 - \sigma_2$	$(q_1-q_2)/$	^{95,97} Mo	Fuler	Reference
Enzyme	Signal Type	g-values	gav	g1-g3	$(g_1 - g_2)'$ $(g_1 - g_3)$	Hyperfine (Mo)	Angles	Reference
		• • • •	1.000	0.005	0.0.50		00.101	
EcYdhV	ferricyanide-	2.008,	1.992	0.035	0.350	79, 149, 11	90, 104,	This work
	oxidized	1.996,					90	
D. D. (GOD		1.973	1			27/1	27/4	1
RCDMSOR	high-g unsplit	1.991,	1.978	0.030	0.250	N/A	N/A	1
	type I	1.983,						
	1.1.1.1.	1.961	1.070	0.024	0.1.00	27/4		1
	high-g unsplit	1.987,	1.978	0.024	0.160	N/A	N/A	1
	type 2	1.983,						
	1.1 1.4	1.963	1.070	0.025	0.242			1
	high-g unsplit	1.989,	1.979	0.025	0.243	N/A	N/A	1
	(glycol)	1.983,						
D.DMCOD	1 1. 4	1.964	1.00	0.020	0.222			2
RSDMSOR	nign-g unsplit	1.99,	1.98	0.030	0.333	IN/A	IN/A	-
	(glycerol)	1.98,						
	high ganlit	1.90	1.007	0.027	0.270	40 112 156	NT/A	3
	nign-g split	1.999,	1.987	0.027	0.370	40, 112, 156	IN/A	5
	(TMAO)	1.989,						
E-DMCOD	mained at	1.972	1.074	0.027	0.407	NT/ A	NT/A	4-6
ECDMSOR	F = 51 mV	1.987,	1.9/4	0.027	0.407	IN/A	IN/A	
	L = -31 mV	1.970,						
FeNarGH	high pH	1.900	1 076	0.027	0.258	07 126 08	N/A	7-9
Leivaion	ingn pri	1.987,	1.970	0.027	0.230	97, 120, 98	11/7	
		1.960,						
	low nH	2 001	1 983	0.037	0.437	N/A	N/A	7-9
	low pri	1 985	1.705	0.057	0.457	11/21	11/21	
		1.964						
<i>Ec</i> FDH(Se)	$\sigma = 2.094$	2.094	2 0 2 9	0 104	0 894	118 71 71	N/A	10
_H	signal	2.001	2.02)	0.101	0.071	110, / 1, / 1	1011	
	518141	1 990						
CnFDH	dithionite-	2.009.	2.001	0.017	0.471	138, 82, 45	11.0.153	11
	reduced	2.001.				,,	,,	
		1.992						
<i>Ws</i> PsrABC	very-high g	2.017,	2.002	0.029	0.481	56,56, 140	N/A	12
	(polysulfide)	2.003,						
	a s ,	1.987						
S.sp.	turnover-	1.990,	1.976	0.033	0.303	122, 68, 143	N/A	13
ArrAB	detected	1.980,						
		1.957						
Chicken	high pH	1.990,	1.970	0.036	0.666	164, 63, 34	0, 14, 22	14
SO		1.966,						
		1.954						
PpSoxCD1	high pH	1.985,	1.965	0.035	0.686	162, 64, 30	0, 21, 41	15
		1.961,						
		1.950						
Bovine XO	very rapid	2.025,	1.977	0.076	0.921	133, 55, 57	8, 36, 0	16
		1.955,						
		1.949						

rapid type 1	1.990,	1.976	0.023	0.826	184, 74, 77	0, 18, 0	16
formamide	1.971,						
	1.967						
desulfo	1.971,	1.963	0.016	0.3110	196, 79, 81	81, 79,	17
	1.966,					196	
	1.954						
formaldehyde	1.991,	1.973	0.040	0.349	159, 69, 156	178, 69,	18, 19
-inhibited	1.977,					67	
	1.951						

N/A not applicable

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Table S5: Fe EXAFS simulation parameters of YdhV.^a

Fe-O,C	Fe-S	Fe-Fe
	N [per Fe] / R [Å]	
1.2 / 2.50	2.1 / 2.23	16/276
3.5 / 2.92	1.5 / 2.38	1.0 / 2. /0

^aParameters correspond to the EXAFS spectrum in Fig. 9 (N, coordination number; R, interatomic distance). The fit error sum, R_F, for reduced distances of 1-3 Å was 10.9 %. A Debye-Waller factor $(2\sigma^2)$ of 0.002 Å² was employed for all shells except the Fe-Fe distance $(2\sigma^2 = 0.005 \text{ Å}^2)$ in the fitting.

YdhV	1	MANGWTGNILRVNLTTGNITL – EDSSKFKSFVGGMGFGYKIMYDEVPPGTKPFDEANKL
BamB	1	MRYAETGYVLEVDLTKGSIERVATDPRDTELYLGGLGTNAKILWDRVPPEVEPFSPENLL
YdhV	59	VFATGPITGSGAPCSSRVNITSLSTFTKGNIVVDAFNGGFFAAQMKFAGYDVITIEGKAK
BamB	61	IFAAGLICGTPATGCNRTIVSTVSPQTKIMAFSM <mark>N</mark> GGFWAPELKYAGYDKIIFRGKSP
YdhV	119	SPVWLNIKDDKVSLEKADFLWGKGTRATTEEIC – RLTSPETCVAAIGQAGENLVPLSGML
BamB	119	ELVYLYINNDKVEIRDASHLKGKGAIETAEIIKKELNEPRAQVAAIGKAGENRVFYASIE
YdhV	178	NSRNH <mark>S</mark> GGAGTGAIMGSKNLKAIAIEGTKGVNIADROEMKSLNDYMMTELIGANNNHVVP
BamB	179	QGRSSA <mark>S</mark> RGGI <mark>GAVMG</mark> DKGLKAVVVRGTKDLCVAKPEEYIGLCNEVLDYIKHREEN-PIP
YdhV	238	STPQSWAEYSDPKSRWTARKGLFWGAAEGGPI <mark>ETGEIPPGNQNTV</mark> GFRTYKSVF
BamB	238	DVMPIL <mark>A</mark> GLG <mark>SPQEMK</mark> VHDEKWHT-ENFNWGN <u>A</u> RTRRKDFWTDEVSH
YdhV	292	DLGPAAEKYTVKMSC <mark>C</mark> HSCPIFCMTQMNIPRVKEFGVPGTGGNTCVANFVHTTIFPNGPK
BamB	284	AWEKTMDKARTRLISCINCPMKCGATISMEGLPTYMMKCFTKLTYTMA
YdhV	352	DFEDKDDGRVIGNLVGLNLFDDYGLWCNYGQLHRDFTYC-YSKGVFKRVLPAEEYAEIRW
BamB	332	AYSDLDFGLRIAQKATEYGLDGFSAPQVMAFAFELLEKGILKDSDF
YdhV	411	DQUEAGDVNFIKDFYYRLAHCVGELSHLADGSYAIAERWNLGEEYWGYAKNKLWSPF
BamB	378	PGLPEGNEERFFYLLDKIVNRDGIGDILANGTYWAAQEIGNGAEDYAHNNIKKHE
YdhV	468	GYPVHHANEASAQVGSIVNCMFNRDCMTHTHINFIGSGLPLKLQREVA
BamB	433	QLPLKLSMLNPIYYLMYCTGEKINI <mark>H</mark> QIEGQFPQAPYPKLEQREAFVEDWIQVPDEKF
YdhV	516	KELFGS-EDAYDETKNYTPINDAKIKYAKWSLLRVCLHNAVTICNWVWPMTVSP-LKSRN
BamB	491	KKIFLEWEPRGEKSMPNFPTVDMCCDIVDWQEMMHYIDDALGCCAGLSSFPLKPPYHIH-
YdhV	574	YRGDLALEAKFFKAITGEDMTQEKLDLAAERIFTLHRAYTVKLMQTKDMRNEHDLICSWV
BamB	550	NYPKFIAAGAGIEMDTEKLKKAAKRYRTLVRAFNIRRGMRR
YdhV	634	FDKDPQIPVFTEGTDKMDRDDMHASLTMFYKEMGWDPQLGCPTRETLQRLGLEDIAADLA
BamB	591	VDEQPPANHWKNRFPELEKELLDSYYKLKGWND-DGIPTKETLDDLGLGYVGDEFI
YdhV	694	AHNLLPA-
BamB	646	KRGILSAG

Figure S1A: Sequence alignment of YdhV and BamBC.

Alignment was carried out with Clustal (1.2.4, <u>http://www.uniprot.org/align/</u>) using amino acid sequences for YdhV from *E. coli* (AKK48365.1) and benzoyl-CoA reductase BamBC (PDB ID 4Z3Y) from *Geobacter metallireducens*. Colors denote: magenta = Cys322 (C) ligand of W in BamBC, green = Thr close to W in BamBC, red = cysteine (C) ligands to the [4Fe-4S] cluster next to the W-cofactor in BamBC, orange = amino acid ligands at the magnesium ion bridging the two MPT units of the W-cofactor in BamBC.



Figure S1B: Crystal structure of BamBC.

(A) Structure (truncated) according to PDB entry 4Z3Y (2.36 Å resolution) with positions of W-cofactor and [4Fe-4S] cluster marked. (B) W-bis-MPT-Mg cofactor with neighboring [4Fe-4S] cluster at ~9 Å distance. (C) W-cofactor with bound or neighboring amino acids marked (an O atom at W was assigned in the crystal structure).



Figure S2: Elemental analysis with TXRF. (A) Phosphorus determination in YdhV protein. To YdhV samples ($1.0 \pm 0.1 \text{ mM}$ protein), a phosphorus standard (Na₃PO₄) was added at increasing concentrations (in addition to a gallium concentration standard), the P contents were determined from TXRF spectra (P K α emission at ~ 2.0 keV, not shown), and P per protein ratios were determined. A linear fit (i.e. extrapolation to zero added P) provided a P per protein content in YdhV of 1.8 ± 2 . (B) Quantification of bromine in YdhV samples (green line, YdhV prepared with Cl⁻; blue line, YdhV prepared with Br⁻; spectra are normalized at the Fe K α emission). K α emission lines of selected elements are marked (unmarked features = K β emission lines). A Ga standard was added to the samples at different concentrations. Element per protein ratios from the TXRF analysis are summarized in Table S2.



Figure S3: **X-Band EPR spectra of apo-YdhV**. (A) apo-YdhV aerobically expressed in the presence of 1 mM Na₂MoO₄, (B) apo-YdhV aerobically expressed without Na₂MoO₄. For each panel, the top spectrum represents the as-isolated protein, the middle spectrum represents sodium dithionite-reduced protein, and the bottom spectrum represents $K_3Fe(CN)_6$ -oxidized protein. Features present in the baseline are enlarged as noted (thin lines). Spectra were recorded at 80 K at 4 mW microwave power, 5 G modulation amplitude and 100 kHz modulation frequency. Spectra have been scaled to represent a YdhV concentration of 1094 μ M. For associated concentrations of Mo and Fe, see Table S1.



Figure S4: **EPR spectral simulation of the Mo(V) signal of ferricyanide-oxidized YdhV at 150 K.** The experimental spectrum (black line) represents ferricyanide-oxidized YdhV as depicted in Figure 7 (panel A, bottom black spectrum), measured at 150 K at 4 mW microwave power, 5 G modulation amplitude and 100 kHz modulation frequency. The spectral simulation (red line) includes two components whereby the Mo isotopes that possess a nuclear spin were treated separately using the simulation toolbox EasySpin. Simulation parameters: *g*: 2.008, 1.996, 1.973 ($g_{av} = 1.992$, anisotropy = 0.0354, rhombicity = 0.3503), *g*-strain: 6.7, 8.2, 6.7 (× 10⁻³), A: (95,97 Mo) [78.8, 148.7, 11.4] MHz, Euler angles: 89.8, 103.5, 89.9 (degrees).



Figure S5: **Power-dependent relaxation behavior of paramagnetic species in YdhV.** Panel depicts the power saturation properties of Mo(V)-associated (squares) and Fe-S cluster-associated (circles) EPR signals recorded at 12 K (10 G modulation amplitude, 100 kHz modulation frequency). Solid squares represent the intensity of the bis-Mo-MPT Mo(V) signals (Figure 7, panel A) recorded in the presence (black squares) or absence (red squares) of K₃Fe(CN)₆ (measured at 334.5 mT). Open squares represent the intensity of the signal of unspecific Mo(V) in apo-YdhV expressed in the presence of molybdate (measured at 335.0 mT; Figure S3, panel A). Solid circles represent the intensity of the EPR signal at 12 K attributable to an Fe-S cluster in YdhV (measured at 336.0 mT; see Figure 8, also Figure S3, panel B). For associated concentrations of Mo and Fe, see Table S1.



Figure S6: Wide-sweep X-Band EPR spectra of YdhV at low temperature. (A) bis-Mo-MPT-loaded YdhV, (B) apo-YdhV aerobically expressed without Na₂MoO₄. Spectra refer to YdhV as-isolated (top), after treatment with 10 mM sodium dithionite (middle), and after treatment with K₃Fe(CN)₆ (bottom), and correspond to the spectra of samples in Figure 1 (panel A) and Figure S1 (panel B). Spectra were recorded at 12 K at 4 mW microwave power, 5 G modulation amplitude and 100 kHz modulation frequency. Spectra in panel A represent a YdhV concentration of 202 μ M, while spectra in panel B represent a YdhV concentration of 662 μ M. The large feature at 250 mT in panel A represents the signal of excess paramagnetic ferricyanide in the samples that is readily observed at 12 K. For associated concentrations of Mo and Fe, see Table S1.



Figure S7: Fe XAS of YdhV. (A) Fe K-edge spectrum. (B) Fourier-transform (FT) of EXAFS spectrum in the inset (black lines, experimental data; blue lines, simulation with parameters in Table S5; the first FT peak reflects Fe-S,O bonds and the second peak Fe-Fe,C distances). The FT was calculated for a k-range of 2-12 Å⁻¹ (cos windows of 10 % at both k-range ends). The XANES shows the typical shape of predominately sulfur-coordinated, tetrahedral iron. The EXAFS Fourier-transform displays two main peak features mainly due to Fe-S and Fe-Fe distances. Simulation of the EXAFS spectrum revealed less than 4 Fe-S bonds (~2.2-2.4 Å), about 1 Fe-O bond (~2.5 Å), and ~1.6 Fe-Fe distances (~2.7 Å), as well as longer Fe-C distances per iron ion (Table S5). The coordination numbers deviate from the values expected for a [4Fe-4S] cluster (4 Fe-S bonds, 3 Fe-Fe distances) or a [2Fe-2S] cluster (4 Fe-S bonds, 1 Fe-Fe distance).