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Structure of the Molybdenum Site in YedY, a Sulfite Oxidase Homologue from *Escherichia coli*

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YedY from *Escherichia coli* is a new member of the sulfite oxidase family of molybdenum cofactor (Moco)-containing oxidoreductases. We investigated the atomic structure of the molybdenum site in YedY by X-ray absorption spectroscopy, in comparison to human sulfite oxidase (hSO) and to a Mo^{IV} model complex. The K-edge energy was indicative of Mo^{V} in YedY, in agreement with X- and Q-band electron paramagnetic resonance results, whereas the hSO protein contained Mo^{VI}. In YedY and hSO, molybdenum is coordinated by two sulfur ligands from the molybdopterin ligand of the Moco, one thiolate sulfur of a cysteine (average Mo−S bond length of ∼2.4 Å), and one (axial) oxo ligand (Mo═O, ∼1.7 Å). hSO contained a second oxo group at Mo as expected, but in YedY, two species in about a 1:1 ratio were found at the active site, corresponding to an equatorial Mo−OH bond (∼2.1 Å) or possibly to a shorter $Mo-O^{-}$ bond. Yet another oxygen (or nitrogen) at a ∼2.6 Å distance to Mo in YedY was identified, which could originate from a water molecule in the substrate binding cavity or from an amino acid residue close to the molybdenum site, i.e., Glu104, that is replaced by a glycine in hSO, or Asn45. The addition of the poor substrate dimethyl sulfoxide to YedY left the molybdenum coordination unchanged at high pH. In contrast, we found indications that the better substrate trimethylamine *N*-oxide and the substrate analogue acetone were bound at a ∼2.6 Å distance to the molybdenum, presumably replacing the equatorial oxygen ligand. These findings were used to interpret the recent crystal structure of YedY and bear implications for its catalytic mechanism.

Introduction

Molybdenum-containing oxidoreductases are found in most organisms and catalyze a wide variety of transformations at carbon, sulfur, and nitrogen atoms, which include the transfer of an oxo group or two electrons to or from the substrate.[1] More than forty molybdenum and tungsten enzymes were identified in bacteria, archaea, plants, and animals to date. With the exception of nitrogenase, which binds the unique Fe−Mo cofactor,[2] all other molybdoenzymes bind the molybdenum cofactor (Moco), a tricyclic pyranopterin with a unique dithiolene group coordinating the molybdenum atom.

The mononuclear molybdenum enzymes are categorized on the basis of the structures of their molybdenum centers, dividing them into three families, each with a distinct active site structure and a different type of reaction catalyzed: the xanthine oxidase (XO) family, the sulfite oxidase (SO) family, and the dimethyl sulfoxide (DMSO) reductase family.[1] The XO family is characterized by an $LMo^{VI}OS(OH)$ core in the oxidized state, with one pterin cofactor (designated as L) coordinated to the metal. These enzymes typically catalyze the hydroxylation of carbon centers. Enzymes of the SO family also coordinate a single pterin cofactor with an $LMo^{VI}O_2$-(S-Cys) core in its oxidized state; the cysteine ligand is provided by the polypeptide. Members of this family (including SO and nitrate reductase) catalyze the transfer of an oxygen atom either to or from the substrate. The DMSO reductase family is diverse in both structure and function, but all members have two pterin cofactors bound to the metal [bis-MGD Moco, where MGD = molybdopterin (MPT)−guanine dinucleotide]. The molybdenum coordination sphere is usually completed by a single Mo═O group with a sixth ligand in the $L_2Mo^{VI}O(X)$ core. The sixth ligand, X, can be a serine, a

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cysteine, a selenocysteine, or a hydroxide and/or water molecule. The reactions catalyzed by members of this family frequently involve oxygen-atom transfer, but dehydrogenation reactions also occur.

Recently, a new member of the SO family has been discovered in *Escherichia coli* and was named YedY.[4] Most *E. coli* enzymes utilize the bis-MGD form of Moco and belong to the DMSO reductase family.[3] In YedY, the Mo-MPT form of Moco was identified.[4] The *yedY* gene was identified in a screening for genes of molybdoenzymes.[4] Compared to molybdoproteins with a known function, the amino acid sequence of YedY is most homologous to the Moco-binding domain II of SO.[4] YedY has a twin-arginine translocation (TAT) signal peptide, which targets the folded protein for translocation across the cytoplasmic membrane.[5] Together with *yedY*, the *yedZ* gene forms an operon.[4] YedZ is a membrane-spanning cytochrome *b* like protein, which probably provides both a membrane anchor and a redox partner for the soluble YedY.[4,6] The YedYZ complex thus constitutes a heme-molybdoenzyme similar to the SO subunits I and II. Orthologues of the YedYZ complex are also present in other Gram-negative bacteria, including many pathogenic strains.[4]

Recently, the crystal structure of YedY was solved at 2.5 Å resolution.[4] The overall fold of the Moco binding site is similar in both YedY and SO, but the substrate binding site in YedY was shown to be different. For accommodation of the negatively charged sulfite/sulfate substrate, SO contains strictly conserved basic residues (Arg138, Arg190, and Arg450 in chicken SO[7]) in its substrate binding pocket, whereas YedY instead shows aromatic amino acid residues (Tyr47, Tyr231, Trp223, and Trp246[4]), forming a cavity at the entrance to the active site. The YedY substrate site thus is more similar to those of the bacterial reductases like DMSO and trimethylamine *N*-oxide (TMAO) reductases.[8,9] Accordingly, YedY shows reductase activity only with substrates such as TMAO and DMSO, and no oxidase activity with sulfite.[4] Substrate screenings revealed catalytic activity generally only with *S*- and *N*-oxides.[6] The turnover rate of YedY, however, is much lower than those of DMSO[10] and TMAO reductases[11] in *E. coli*. Hence, the function and substrate of YedYZ in *E. coli* still remain unknown.

SO catalyzes the two-electron oxidation of sulfite to sulfate at the molybdenum atom of Moco.[12] SO usually is isolated in its oxidized state containing Mo^{VI}, and during catalytic turnover, the metal is reduced to Mo^{IV}.[13] In contrast, purified YedY shows an electron paramagnetic resonance (EPR) signal from Mo^{V} in its as-isolated state.[6] The midpoint potential (E_m) of the $Mo^{V} \rightarrow Mo^{VI}$ oxidation seems to be unusually positive, because even at +350 mV the Mo^{V} EPR signal was still observed.[6] Spectroscopy results and density functional theory calculations have been interpreted as suggesting that the geometry of the molybdenum coordination may modulate E_m of the Moco.[14]

The enzymatic activity and substrate preference of Moco enzymes are determined by the specific coordination of the molybdenum atom. To gain mechanistic information, high-resolution structural information on the Mo site is required. In the crystal structure of YedY, the molybdenum ion is coordinated by two sulfur atoms of MPT, the thiolate of Cys102, and one oxo group.[4] A urea molecule from the crystallization buffer was found in the potential substrate site. The electron density around the fifth molybdenum coordination site could not be assigned unambiguously,[4] but a second oxo group and a water molecule linking it to urea, or a water molecule directly bound to urea, have been considered.

Previous X-ray absorption spectroscopy (XAS)[13,15,16] and crystallographic[7,17] studies have provided detailed insights into coordination of the molybdenum atom in SO, in both its oxidized (Mo^{VI}) and reduced (Mo^{IV}) states. In the present investigation, XAS at the Mo K-edge and in addition X- and Q-band EPR spectroscopy were employed to determine the ligand environment of the molybdenum atom in YedY in the presence and absence of substrate analogues, in comparison to the situation in human SO (hSO) and a Mo^{IV} model compound.

Materials and Methods

Cloning, Expression, and Purification of YedY. The gene encoding *E. coli* YedY was isolated from the *E. coli* K12 genome.[18] The published gene sequence was used to design primers that permitted cloning into the *Nde*I and *Sal*I sites of the expression vector pTrcHis.[19] The resulting plasmid, designated as pSR8. For heterologous expression in *E. coli*, pSR8 was transformed into *E. coli* TP1000 cells,[20] containing a deletion in the *mobAB* genes responsible for Moco dinucleotide formation.

For the expression of either YedY (pSR8) or hSO (pTG718[19]) in TP1000, a Luria−Bertani medium containing 1 mM Na_2MoO_4, 20 μM isopropyl thio-β-D-galactoside, and 150 μg/mL ampicillin was inoculated with 2 mL/L of an overnight culture and incubated at 30 °C for 24 h. After centrifugation, the cell

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pellet was resuspended in a phosphate buffer (50 mM NaH_2PO_4 and 300 mM NaCl, pH 8.0). Cells were lysed by two passages through a Disrupter TS Series bench top (Constant Systems), and the cleared lysate was applied to 0.75 mL for YedY and 0.9 mL for hSO nickel nitrilotriacetate resin (Qiagen) per liter of culture. The column was washed with 20 column volumes of a phosphate buffer, one containing 10 mM imidazole and the other 20 mM imidazole. Protein was eluted with a phosphate buffer containing 250 mM imidazole. YedY was dialyzed overnight against 50 mM NaH_2PO_4 and 300 mM NaCl, pH 8.0, and hSO against 100 mM Tris/HCl, pH 7.2.

For the preparation of extended X-ray absorption fine structure (EXAFS) samples, YedY was concentrated to 1.2 mM using an Amicon ultrafiltration device (10 000 MWCO). The DMSO-, TMAO-, or acetone-containing YedY sample was incubated with 1.5 M DMSO, TMAO, or acetone for 20 min, and unbound DMSO was removed by gel filtration using a Nick column (GE Healthcare).

The activity of YedY with TMAO and DMSO as substrates under different pH levels was measured as previously described.[4,10] The assays were performed in a phosphate−citrate buffer in a pH range from 4 to 8, containing 0.4 mM benzyl viologen, 7.5 mM TMAO, or DMSO as substrates and 45 nM YedY. The assay did not work at pH values lower than 4.

Complex Synthesis and Characterization. The synthesis of compound **1** was done using Schlenk-line techniques under a pure nitrogen atmosphere. Solvents were degassed by bubbling through nitrogen for 1 h. $K_3Na[MoO_2(CN)_4]\cdot 6H_2O$ and 4,5-dimethylbenzene-1,2-dithiol were synthesized according to the respective literature procedures. $[PPh_4]_2[MoO(xdt)_2]$:($[PPh_4]_2$)-$K_3Na[MoO_2(CN)_4]\cdot 6H_2O$ (0.86 g, 1.62 mmol) and NaOH (0.23 g, 5.87 mmol) were dissolved in H_2O (20 mL). 4,5-Dimethylbenzene-1,2-dithiol (0.50 g, 2.94 mmol) in MeOH (20 mL) was added, and a color change from blue to red was observed. The reaction mixture was refluxed for 20 min, generating a dark-red solution, which was cooled and filtered before a solution of PPh_4Cl (1.10 g, 2.94 mol) in MeOH (5 mL) was added. The reaction mixture was stirred for another 1 h at room temperature. Treatment with additional H_2O (15 mL) resulted in precipitation of an orange-brown solid. The product was recrystallized from acetonitrile by the addition of Et_2O through vapor diffusion. For further analytic results and crystal data of **1** see the Supporting Information (Figure S1 and Table S1).

EPR Spectroscopy. EPR spectra of as-isolated YedY were recorded on a Bruker ER 300 spectrometer equipped with an ER 035 M gaussmeter and a HP 5352B microwave frequency counter and either a Bruker ST 4102 X-band or QTN 5102 Q-band resonator. The temperature was controlled using a Bruker ER 4111 VT variable-temperature unit and a liquid-nitrogen cryostat. Spectra were collected at 150 K with the following instrument settings: microwave frequency, 9.4565 GHz (X-band) or 34.0316 GHz (Q-band); microwave power, 1.0 mW (X-band) or 4 mW (Q-band); modulation amplitude, 0.4 mT (X- and Q-bands). The Q-band spectrum was recorded as the average of 16 separate scans. Simulations were performed using the *EasySpin 3.1.0* software package.[21] Anisotropic hyperfine splittings due to ~25% $^{95}Mo/^{97}Mo$ contributions to the spectra and Euler angles relating the **g** and **A** tensor frames were obtained from simulations of a single species at both the X- and Q-bands; these values were subsequently fixed for the two-species simulation, and only the *g* values and line-width parameters were allowed to converge freely.

XAS. XAS at the Mo K-edge was performed at the Synchrotron SOLEIL (Paris, France) at the SAMBA bending-magnet beamline using a double-crystal Si(111) monochromator for scanning of the excitation energy and two palladium-coated mirrors in grazing incidence mode for focusing of the X-ray beam (spot size ~2.5×1 mm^2) and for harmonics rejection. The energy axis was calibrated (accuracy ±0.1 eV) using the first inflection point at 20 003.9 eV in the simultaneously measured absorption spectrum of a molybdenum foil as a standard. Fluorescence-detected XAS spectra were measured using an energy-resolving seven-element solid-state germanium detector (Canberra), which was shielded by 10 μm zirconium foil against scattered X-rays. The total incoming count rate was kept well below 20 000 s^{-1} to avoid detector saturation. Samples were held in a laboratory-built liquid-helium cryostat at 20 K. XAS spectra (scan range of 19 850−20 750 eV, i.e., up to $k = 14$ $Å^{-1}$; 2 scans per sample spot) were corrected for detector deadtime and averaging (8−10 scans per sample), normalization, extraction of EXAFS oscillations, and conversion of the energy scale to the wave-vector scale (*k* scale) were performed as previously described.[22] k^3-weighted EXAFS spectra were simulated (S_0^2 = 1.0) by a least-squares procedure using phase functions calculated with *FEFF7*,[23] and Fourier transforms (FTs) were calculated using the in-house software *SimX*.[24,25] E_0 was refined to 20 014 ± 2 eV in the fit procedure. The fit quality was judged by calculation of the Fourier-filtered *R* factor (R_F).[26] The preedge structure of X-ray absorption near-edge structure (XANES) spectra was isolated by subtracting a polynomial spline from the main K-edge rise using the software *XANDA*.[27] K-edge energies reflect values at 50% of the normalized edge absorption (edge half-height).

Results

Molybdenum Oxidation State. The XANES region (K-edge) of XAS spectra is sensitive to the metal oxidation state and to the number, chemical identity, and geometry of first-sphere metal ligands.[28] Figure 1A compares XANES spectra of as-isolated YedY protein, of hSO protein containing Mo^{VI}, and of a synthetic Mo^{IV} complex ($[MoO(xdt)_2]^{2-}$ with xdt = 4,5-methylbenzene-1,2-dithiolate; further on denoted as **1**). The K-edge energies of **1** and hSO were 20 012.0 and 20 014.2 eV, respectively (Table 1). The edge of YedY was in between (20 013.1 eV) and thus shifted by +1.1 eV relative to **1** and by −1.1 eV relative to hSO. For a one-electron oxidation of molybdenum, a shift by 1−1.5 eV to higher energies was expected, for a mixed S/O coordination environment.[29] Thus, the edge energy of YedY (Figure 1A, lower inset) suggested that its molybdenum atom indeed was in the Mo^V oxidation state, in agreement with EPR experiments.[6,14]

The preedge peak in the XANES spectrum (Figure 1B) arises from formally dipole-forbidden 1s → 4d electronic transitions,[28] and distortion from the octahedral molybdenum coordination geometry, i.e., due to the presence of short Mo=O bonds, causes enhanced p−d orbital mixing and increases the intensity of the preedge feature. The preedge peak of YedY was smaller and shifted by ~1 eV

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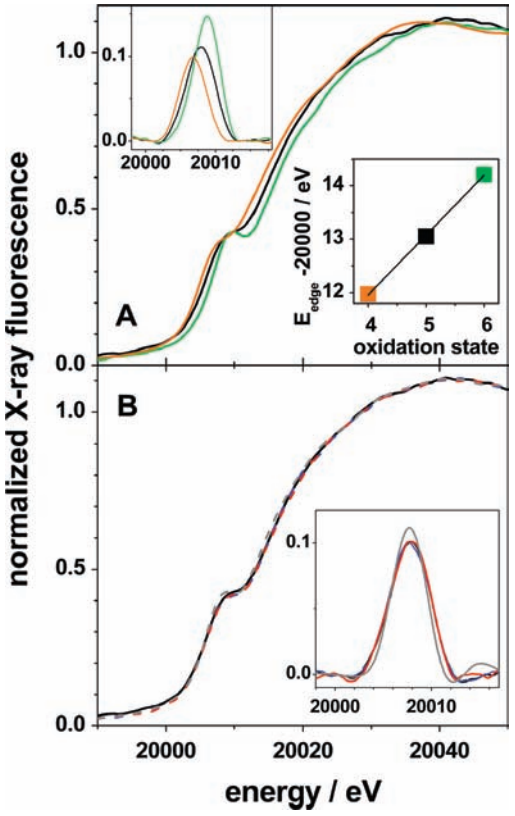

Figure 1. (A) Mo XANES spectra of YedY (black), hSO (green), and Mo^{IV} complex **1** (orange): upper inset, respective isolated preedge peak features; lower inset, respective K-edge energies. (B) XANES of YedY alone (black) and of YedY in the presence of DMSO (red), TMAO (gray), and acetone (blue) and the corresponding isolated preedge peaks (inset).

Table 1. Mo K-Edge Energies and Preedge Peak Energies and Areas from XANES Spectra[a]

sample	E_{edge} [eV]	$E_{preedge}$ [eV]	$A_{preedge}$ [r.u.]
YedY	20 013.1	20 007.8	0.74
YedY + DMSO	20 013.2	20 007.8	0.75
YedY + acetone	20 013.3	20 007.7	0.75
YedY + TMAO	20 012.8	20 007.7	0.69
hSO (Mo^{VI})	20 014.2	20 008.7	1.00
1 (Mo^{IV})	20 012.0	20 006.8	0.67

[a] E_{edge} (±0.1 eV) refers to the 50% level of normalized fluorescence; $E_{preedge}$ (±0.1 eV) is for the preedge peak maximum obtained by Gaussian simulations; $A_{preedge}$ results from integration of the respective spectra, and values are given relative to the area of the hSO spectrum (Figure 1).

to lower energies compared to that of hSO but larger and shifted by ~1 eV to higher energies compared to that of **1** (Figure 1A, upper inset, and Table 1). There are two Mo=O bonds in hSO[13,15,16,30] and one in **1** [Supporting Information (SI), Figure S1]. Thus, the preedge peak of YedY suggested that its molybdenum atom was coordinated by less than two, but more than one, oxo groups. A similar result was obtained in the EXAFS analysis (below).

XANES spectra of YedY samples that contained the weak substrate DMSO, the better substrate TMAO, and the substrate analogue acetone showed only very minor differences in edge shapes and energies (Figure 1B and Table 1). Accordingly, major changes in the first coordination shell of molybdenum in the presence of these additives were not detectable.

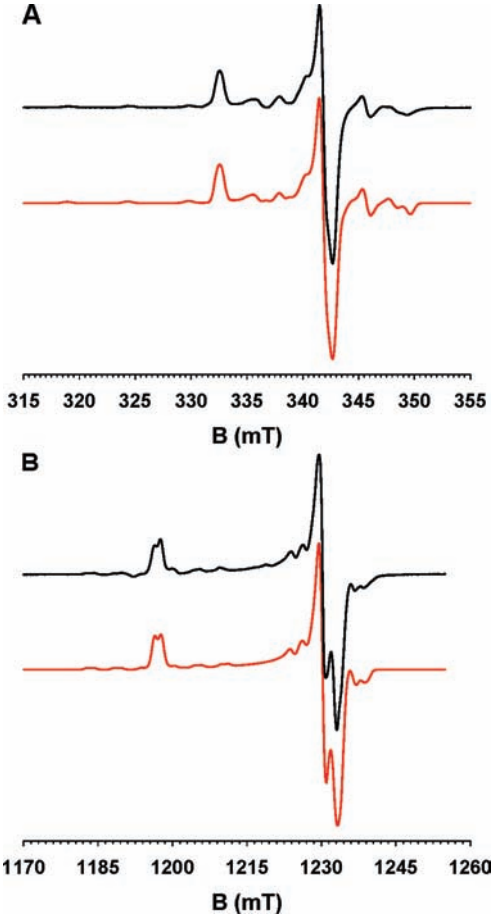

Figure 2. Mo^{V} EPR spectra of YedY. Shown are X-band (A) and Q-band (B) spectra (black) and respective simulations (red). Simulation results are summarized in Table 2.

The oxidation state of molybdenum was verified to be largely Mo^{V} by EPR spectroscopy. We confirm the X-band EPR spectra of YedY reported previously,[14] indicating an approximately axial Mo^{V} EPR signal with poorly resolved g_2 and g_3 tensors (Figure 2A). We have also recorded the Q-band EPR spectrum (Figure 2B) to improve the resolution of the **g** tensor. The spectrum unambiguously demonstrated that the Mo^{V} signal in YedY is indeed rhombic. Simulation parameters of the spectra obtained at the two microwave frequencies (Figure 2, red lines) are reported in Table 2 and are in good agreement with the previously published values.[14] We were able to extend our analysis to include the hyperfine splittings due to ~25% $^{95}Mo/^{97}Mo$ contributions to the spectra and Euler angles relating the **g** and **A** tensor frames. Q-band simulations also show the presence of two species in approximately a 40:60 ratio, based on the relative intensities of the g_1 features. Two species, presumably due to a protonation equilibrium at a molybdenum-bound oxygen, were also suggested by XAS analysis (below).

Mo−Ligand Distances from EXAFS Simulations. From curve-fitting of EXAFS spectra (Figure 3), the numbers of ligand species of the molybdenum atom and precise Mo−ligand bond lengths were determined. FTs of the spectra of YedY and hSO showed two main peaks due to Mo−O

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Table 2. EPR Simulation Parameters for As-Isolated YedY

	g_1/g_1'	g_2/g_2'	g_3/g_3'	$A_1{}^a$	$A_2{}^a$	$A_3{}^a$	α^a	β^a	γ^a
X band	2.033/2.030	1.977/1.976	1.972/1.970	155.3	61.4	59.0	78.6	19.5	−68.8
Q band	2.032/2.030	1.977/1.976	1.972/1.970						

[a] Anisotropic hyperfine splittings (A, in MHz) and Euler angles (α, β, γ; in deg) were obtained from simulations of a single species in both X- and Q-bands and were subsequently fixed for the two-species simulation.

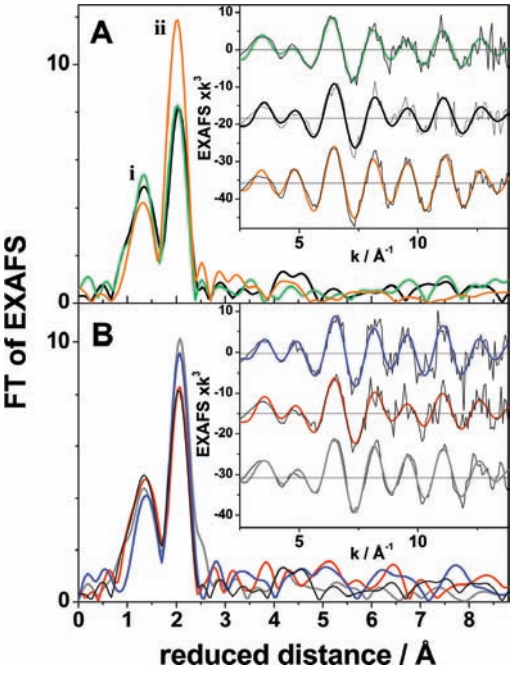

Figure 3. (A) Mo EXAFS spectra of YedY (black), hSO (green), and complex **1** (orange). FTs of the k^3-weighted EXAFS oscillations in the inset: thin black lines, experimental data; thick lines, simulations with parameters in Table 3 (fits b, g, and p). (B) FTs of EXAFS spectra of YedY alone (thin black) and in the presence of DMSO (red), TMAO (gray), and acetone (blue). EXAFS spectra in the inset: thin black lines, experimental data; thick lines, simulations with parameters in Table 3 (fits i, l, and o). FTs were calculated for k values of 2−14 Å^{-1} using cos^2 windows extending over 10% at both k-range ends. Spectra in the insets are drawn on the same scale and vertically displaced for better comparison.

(i) and Mo−S (ii) interactions and were quite similar, suggesting an overall similar structure of their molybdenum sites (Figure 3A). The ratio of the FT peak amplitudes of **1** was different, as expected for one Mo=O and four Mo−S bonds in the model complex (SI, Figure S1).

The EXAFS spectrum of hSO was well simulated (R_F < 10%) already with one sulfur shell including three sulfur ligands (mean Mo−S distance of 2.40 Å) and one oxygen shell with two oxygen atoms (mean Mo−O distance of 1.72 Å) (Table 3, fits a and b). The short oxygen distances suggested two Mo=O bonds. Two oxygen shells did not improve the fit quality (not shown). These results are consistent with previous XAS data of oxidized hSO,[13,16] which also have shown that its Mo^{VI} carries two oxo groups.

The YedY EXAFS spectrum could also be simulated with one sulfur shell and one oxygen shell (Table 3, fits c and d), but the fit quality was much lower than that for hSO (R_F of 18−24%). The use of two oxygen shells (bond lengths of ∼1.7 and ∼2.1 Å; fits e and f) improved the fit, in particular for the use of respective noninteger coordination numbers (fit f). The tentative use of two sulfur shells in the simulation of a YedY spectrum, which was measured to a higher k value of 16 Å^{-1} (SI, Figure S2), yielded one long (2.45 Å) and two shorter Mo−S distances (2.36 Å). The difference in the Mo−S bond lengths (ΔR) was close to the EXAFS resolution limit of ∼0.1 Å, as calculated according to $\Delta R = \pi/2k$ with k = 16 Å^{-1}.

Table 3. Simulation Results of Mo EXAFS Spectra[a]

sample	fit	shell	N_i [per Mo]	R_i [Å]	$2\sigma^2$ [Å^2]	R_F [%]
hSO	a	Mo=O	2.0	1.72	0.008	5.7
		Mo−S	3.3	2.40	0.008	
	b	Mo=O	2[b]	1.72	0.008	9.0
		Mo−S	3[b]	2.40	0.007	
YedY	c	Mo=O	1.4	1.71	0.004	18.0
		Mo−S	3.6	2.40	0.010	
	d	Mo=O	2[b]	1.71	0.008	24.3
		Mo−S	3[b]	2.40	0.008	
	e	Mo=O	1[b]	1.72	0.001	22.2
		Mo−OH	1[b]	2.17	0.010	
		Mo−S	3[b]	2.41	0.008	
	f	Mo=O	1.4	1.71	0.004[b]	17.2
		Mo−OH	0.6	2.12	0.004[b]	
		Mo−S	3[b]	2.41	0.008	
	g	Mo=O	1.5	1.70	0.004[b]	10.8
		Mo−OH	0.5	2.10	0.004[b]	
		Mo−S	3[b]	2.39	0.009	
		Mo−O/N	1[b]	2.59	0.002	
YedY + DMSO	h	Mo=O	1.5	1.72	0.004[b]	15.8
		Mo−OH	0.5	2.09	0.004[b]	
		Mo−S	3[b]	2.41	0.007	
	i	Mo=O	1.5	1.71	0.004[b]	10.5
		Mo−OH	0.5	2.06	0.004[b]	
		Mo−S	3[b]	2.40	0.010	
		Mo−O/N	1[b]	2.60	0.002	
YedY +acetone	j	Mo=O	1.1	1.71	0.004[b]	15.9
		Mo−OH	0.4	2.12	0.004[b]	
		Mo−S	3[b]	2.42	0.006	
	k	Mo=O	1.1	1.70	0.004	7.6
		Mo−S	3[b]	2.40	0.008	
		Mo−O/N	1.9	2.57	0.002[b]	
	l	Mo=O	1[b]	1.70	0.003	6.8
		Mo−OH	0.3	2.09	0.004[b]	
		Mo−S	3[b]	2.40	0.008	
		Mo−O/N	1.7	2.57	0.002[b]	
YedY +TMAO	m	Mo=O	1.2	1.72	0.004[b]	22.5
		Mo−OH	0.6	2.17	0.004[b]	
		Mo−S	3[b]	2.42	0.005	
	n	Mo=O	1.3	1.71	0.006	14.3
		Mo−S	3[b]	2.40	0.007	
		Mo−O/N	1.7	2.59	0.002[b]	
	o	Mo=O	1.3	1.71	0.006	11.8
		Mo−S	3[b]	2.39	0.007	
		Mo−O/N	1.7	2.59	0.002[b]	
		Mo−O/N	1.0	2.86	0.002[b]	
complex ***1***	p	Mo=O	1[b]	1.70	0.003	12.4
		Mo−S	4[b]	2.39	0.005	

[a] N_i, coordination number; R_i, Mo−ligand distance; $2\sigma_i^2$, Debye−Waller factor. The R_F value[26] was calculated over reduced distances of 1−2.6 Å in FTs. [b] Values that were fixed at the given numbers in the simulations. The total numbers of first-sphere ligands to molybdenum (R < 2.7 Å) were restrained to values of ≤6.

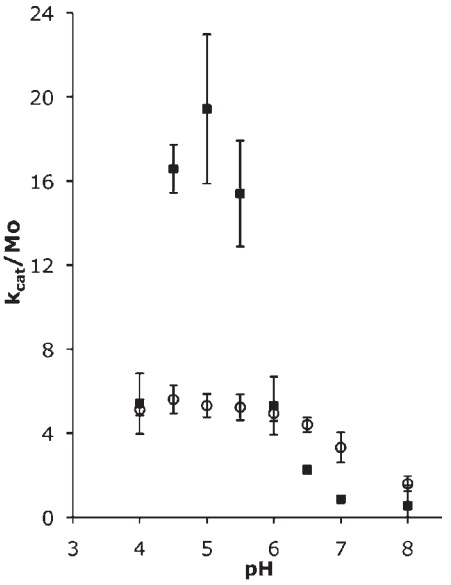

Figure 4. Activity of YedY as a function of the pH. The pH optimum of YedY was determined by analysis of k_{cat} (s^{-1}) in a phosphate−citrate buffer in a pH range from 4.0 to 8.0, with TMAO (squares) or DMSO (open circles) as substrates. The k_{cat} values for both substrates are given per molybdenum atom of the active portion of YedY and show similar ratios at pH 5 as previously shown.[4]

The two different first-sphere oxygen ligands suggested a more heterogeneous molybdenum coordination pattern in YedY compared to hSO. A simulation of quality similar to that for the hSO spectrum was achieved for YedY by adding a third oxygen/nitrogen shell to the simulation (fit g), with a Mo−O/N distance of ∼2.6 Å, at the limit of what may be considered as a true Mo−O/N bond. The respective oxygen/nitrogen atom may belong to a water molecule or to an amino acid side chain. A water molecule at a ∼2.8 Å distance to molybdenum was modeled in the crystal structure of YedY.[4] The coordination numbers of the shorter Mo−O bonds suggested that close to 50% each of the YedY protein contained either two short bonds or one short bond and a longer oxygen interaction. Tentative splitting of the ∼1.7 Å interactions into two shells resulted in one Mo═O bond of 1.69 Å and ∼0.5 bonds of 1.75 Å, but the distance difference was at the resolution limit (see above). A possible interpretation of two short Mo−O interactions was that the p*K* of the OH group was close to a pH of 8 in the sample so that Mo−OH (∼2.1 Å) was present in about half of the protein and the deprotonated form, Mo−O^- (1.75 Å), in the remainder. Mo−O^- ligation (1.78 Å) has also been observed for the Moco of XO at pH 10.[31]

The addition of DMSO as a relatively poor substrate to YedY samples did not affect the EXAFS (Figure 3B) because the FTs of the spectra of YedY and YedY + DMSO were very similar, in agreement with XANES analysis. The YedY + DMSO spectrum could be simulated by using parameters similar to those for the YedY spectrum (Table 3, fit h). In particular, the same three oxygen ligands (at ∼1.7, ∼2.1, and ∼2.6 Å) were also observed (fit i). Analysis of the pH dependence of YedY showed that the protein is inactive at a pH of 8.0 (Figure 4). Thus, at high pH, DMSO was not bound at the active site. The pH-depended enzyme activity measurements showed that YedY has a pH optimum of about 5.0 (Figure 4). EXAFS spectra could not be recorded at lower pH because the protein precipitated at this pH during up-concentration for XAS samples.

In contrast, after the addition of the substrate analogue acetone to YedY, the FT peak at ∼1.5 Å was narrower, lower in amplitude, and shifted to longer distances, whereas the amplitude of the ∼2.2 Å peak was larger and the peak also was slightly shifted to longer distances, compared to the spectrum of YedY alone (Figure 3B). These features suggested coordination changes at molybdenum in the presence of acetone. The EXAFS simulations revealed that only one short Mo−O interaction of ∼1.7 Å length was detectable for the YedY−acetone spectrum and a ∼2.1 Å Mo−OH distance with low coordination number (Table 3, fits j, l). The best fits (k and l) also included a Mo−O/N distance of ∼2.6 Å with an increased coordination number close to 2 compared to YedY alone. The additional Mo−O/N bonds of ∼2.6 Å in the YedY−acetone sample could reflect an acetone molecule at the active site in more than half of the YedY protein, which potentially replaced the molybdenum-bound O^- group but not the OH species (fit l). However, acetone did not act as a substrate or inhibitor at acidic pH values under which the protein was active because the addition of 10-fold higher concentrations of acetone with respect to DMSO did not change the activity of YedY (data not shown). Hence, the pH of 8 used for the EXAFS studies might enable the binding of acetone, although the enzyme under these conditions showed no catalytic activity (Figure 4).

The addition of the better substrate TMAO to YedY changed the FT of the EXAFS spectrum in a manner similar to that in the case of acetone addition; i.e., the amplitude of the first FT peak decreased and that of the second FT peak increased (Figure 3B). Also, the YedY + TMAO spectrum was well simulated using close to two Mo−O/N bonds of ∼2.6 Å (Table 3, fit n, o). Apparently, TMAO was bound at a similar and relatively long distance to molybdenum as acetone. However, a Mo−OH ligand was not required for simulation of the YedY−TMAO spectrum, but a shell of light scatterers at ∼2.9 Å significantly improved the fit (fit o). The latter shell may be due to the nitrogen and carbon atoms of TMAO in the second coordination sphere of molybdenum.

Discussion

Mo^V Oxidation State. The as-isolated YedY possesses molybdenum in the Mo^V oxidation state, as indicated by the XAS data and by X- and Q-band EPR results (see refs 6 and 14 and this work), whereas in oxidized hSO, a Mo^{VI} ion is present. Our EPR results at both X- and Q-bands confirm the Mo^V spectrum of YedY to be rhombic (although nearly axial), and the Q-band spectrum of the as-isolated protein at pH 8 provides clear evidence for the presence of two individual species with **g** tensor principal values of $g_1/g_1' = 2.033/2.030$, $g_2/g_2' = 1.977/1.976$, and $g_3/g_3' = 1.972/1.970$ in approximately a 40:60 ratio; the g_2 and g_3 components of the signal are adequately resolved only at the Q-band. Two species were also apparent in the

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XAS analysis. By combining the X- and Q-band data, we were able to determine with a high degree of confidence all three Euler angles, which describe the noncoincidence of the **g** and **A** tensor frames. The 19.5° angle (β) is in good agreement with the assignment made in ref 14 and with the 18° and 14° angles of the low-pH and high-pH forms of chicken SO, respectively.[32] The 78.6° (α) and −68.8° (γ) angles, however, appear to be quite distinct, indicating some differences in the active sites of YedY and SO.

A particularly positive midpoint potential (E_m > 350 mV) of molybdenum has been revealed in redox titrations of YedY[6,14] and is suggested to be related to an altered coordination geometry compared to hSO.[14] A particularly covalent Mo−S_{Cys} bond to Mo^V in YedY was inferred from the strong thiolate-to-molybdenum ($S^v_{Cys} \rightarrow Mo_{x^2-y^2}$) ligand-to-metal charge-transfer band in the magnetic circular dichroism spectrum.[14] A more apparent reason for the very positive E_m of molybdenum in YedY could be altered hydrogen-bonding properties of the site. In YedY, a glutamate (Glu104) is at the position where a glycine is found in hSO, which is believed to provide space for binding of the SO_3^{2-} substrate.[7,17] In YedY, the neighboring Glu104 and Asn45 may be involved in a hydrogen-bonded network, also including water molecules and direct molybdenum ligands (Figure 5A). An oxygen (or nitrogen) atom at ∼2.6 Å to molybdenum was indeed found in EXAFS analysis, and in the crystal structure, a water molecule was placed at ∼2.8 Å to molybdenum (Figure 5A). It is tempting to speculate that Glu104 and Asn45 may form a hydrogen bond to such a water molecule, which, in turn, is hydrogen-bonded to an O(H) ligand of molybdenum. Alternatively, an amino acid oxygen or nitrogen atom, i.e., from Glu104 or Asn45, may be at ∼2.6 Å to molybdenum and, e.g., hydrogen-bonded to an O(H) ligand of the metal. Hydrogen bonding of molybdenum ligands would be expected to raise the E_m value of the molybdenum site and thus could contribute to the preference for the Mo^V oxidation state in YedY compared to SO with MoIV after isolation.

Structure of the Molybdenum Site. XAS analysis revealed an overall similar structure of the molybdenum site in YedY and hSO with respect to the two sulfur ligands from the MPT moiety of Moco and the S_{Cys} ligand. Differences in molybdenum coordination were observed for the two oxygen atoms in the first shell (Figure 5). The molybdenum in oxidized hSO carried two oxo groups, as was previously found.[16] In the crystal structure of YedY, the axial oxygen ligand of molybdenum clearly was assigned to an oxo group (Figure 5A), but the nature of the equatorial oxygen ligand remained unclear.[4] The typical Mo=O bond length of 1.7 Å found by XAS confirms one (axial) oxo group at the molybdenum. We attribute the second oxygen ligand, which thus should be in the equatorial position, to either O^- (average Mo−O^- bond length of 1.77 Å[31]) or OH (Mo−OH bond length of ∼2.1 Å) (Figure 5B). Possibly the pH of 8 in the samples was close to the p*K* of the OH group so that its deprotonated state (O^-) was present in about half of the YedY protein. The presence of two species was also observed in the EPR data. In XO, a similar pH-dependent OH/O^- equilibrium of a molybdenum ligand has been deduced

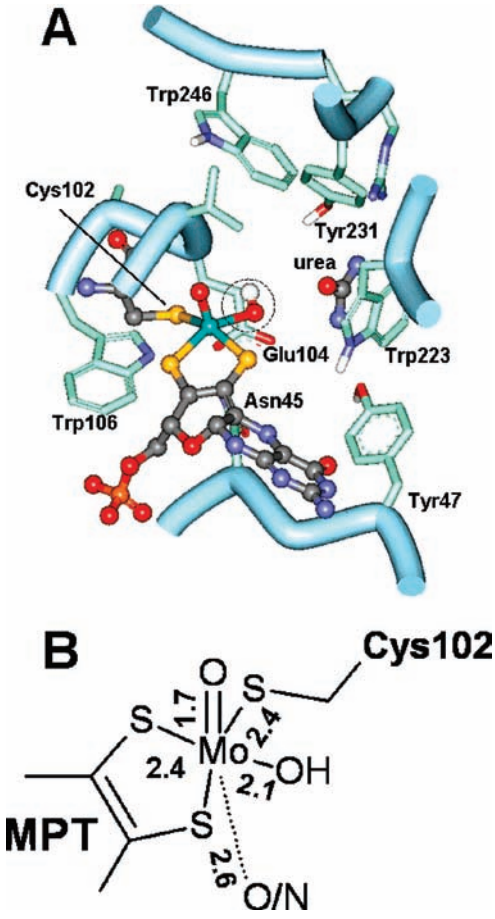

Figure 5. (A) Moco and substrate binding cavity in the crystal structure of YedY.[4] The Moco, urea molecule and amino acids forming the substrate cavity are shown in a ball-and-stick respective stick representations. Color code: C, cyan/gray; O, red; N, blue; S, yellow; P, orange; Mo, green. The dashed circle encompasses a water molecule at ∼2.8 Å to molybdenum. Glu104 is visible below the molybdenum atom, and Asn45 hides behind the pterin. For further details, see the text. (B) Structural model of the Mo^V site of YedY in agreement with the XAS data. Mo−ligand distances are given in angstroms. The equatorial oxygen ligand seems to be an OH or O^- group in each ∼50% of the protein at pH 8, and this oxygen ligand may be replaced by a substrate molecule. The oxygen/nitrogen ligand at ∼2.6 Å to molybdenum may possibly belong to the carboxylic group of Glu104 or the amide group of Asn45 or to a water molecule.

from XAS measurements.[31] Also in the hSO, the equatorial ligand position is occupied by a ligand that changes its protonation stage during the catalytic cycle (Mo^{VI}=O, Mo^VOH, and $Mo^{IV}OH_2$),[13,16] but in this enzyme for the same oxidation state, no deprotonation has been observed up to a pH of 9.[13] The equatorial oxygen ligand apparently is involved in the tuning of the catalytic activity in a variety of Moco enzymes.[33]

In the crystal structure of YedY, the equatorial oxygen ligand points toward the substrate binding site. At pH 8 of the XAS samples, the enzymatic activity of YedY is low and the pH optimum is observed around pH values of 5.0. Thus, YedY seems to be in an inactive form under the XAS conditions. Unfortunately, YedY precipitated at lower pH at the concentrations required for XAS. In previous studies, the shape of the EPR spectrum of Mo^V (in hSO) strongly depended on the pH and salt concentration in the

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sample,[34−36] and pulsed EPR revealed exchangeable protons close to the equatorial oxygen ligand.[37] Thus, the protonation state of the equatorial oxygen ligand in YedY may be crucial for the hydrogen-bonding properties, which control the molybdenum oxidation state and modulate the Mo−substrate interactions.

DMSO and TMAO are both functional substrates, although the catalytic activity at pH 5 is 4 times higher for TMAO. However, at a pH of 8 of the XAS samples, the activities for both substrates were similar and rather low. EXAFS analysis revealed the binding only of TMAO and the substrate analogue acetone at ~2.6 Å distance to molybdenum but not of DMSO. One option for the diverging binding behavior may be different sample preparation procedures, i.e., removal of weakly bound DMSO during the gel filtration procedure. In any event, TMAO and acetone likely replace the equatorial oxygen ligand at molybdenum. The EXAFS results could be interpreted as suggesting that TMAO replaces the respective OH group, whereas acetone perhaps is more likely to replace its deprotonated form, but further investigations are required for unambiguous assignments. Thus, the nature of the equatorial oxygen ligand may be important for both the substrate specificity and activity.

In summary, although YedY and hSO belong to the same family of molybdoenzymes, the differences in the structure of its molybdenum site may be related to the diverging activity and substrate specificity of YedY. The presence of Glu104 and Asn45 close to molybdenum may establish a hydrogen-bonded network, involving the equatorial hydroxyl ligand of molybdenum and a nearby water molecule, which, in turn, favors the Mo^{V} oxidation state and governs interaction of the $Mo-OH/O^{-}$ species with the substrate (Figure 5). This substrate may be expected to be oxidized in single-electron abstraction steps, involving the reduction of Mo^{V} to Mo^{IV} and electron transfer from YedY to its counterpart, the YedZ protein in the membrane.

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Supporting Information Available: Synthesis details, Table S1, and Figures S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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