

Sulfite:Cytochrome *c* Oxidoreductase from *Thiobacillus novellus*

PURIFICATION, CHARACTERIZATION, AND MOLECULAR BIOLOGY OF A HETERODIMERIC MEMBER OF THE SULFITE OXIDASE FAMILY*

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Direct oxidation of sulfite to sulfate occurs in various photo- and chemotrophic sulfur oxidizing microorganisms as the final step in the oxidation of reduced sulfur compounds and is catalyzed by sulfite:cytochrome *c* oxidoreductase (EC 1.8.2.1). Here we show that the enzyme from *Thiobacillus novellus* is a periplasmically located $\alpha\beta$ heterodimer, consisting of a 40.6-kDa subunit containing a molybdenum cofactor and an 8.8-kDa monoheme cytochrome c_{552} subunit (midpoint redox potential, $E_{m8.0} = +280$ mV). The organic component of the molybdenum cofactor was identified as molybdopterin contained in a 1:1 ratio to the Mo content of the enzyme. Electron paramagnetic resonance spectroscopy revealed the presence of a sulfite-inducible Mo(V) signal characteristic of sulfite:acceptor oxidoreductases. However, pH-dependent changes in the electron paramagnetic resonance signal were not detected. Kinetic studies showed that the enzyme exhibits a ping-pong mechanism involving two reactive sites. K_m values for sulfite and cytochrome c_{550} were determined to be 27 and 4 μM , respectively; the enzyme was found to be reversibly inhibited by sulfate and various buffer ions. The *sorAB* genes, which encode the enzyme, appear to form an operon, which is preceded by a putative extra-cytoplasmic function-type promoter and contains a hairpin loop termination structure downstream of *sorB*. While SorA exhibits significant similarities to known sequences of eukaryotic and bacterial sulfite:acceptor oxidoreductases, SorB does not appear to be closely related to any known *c*-type cytochromes.

Sulfite:acceptor oxidoreductases (SOR)¹ (often termed sulfite oxidases although the physiological electron acceptor appears

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF154565.

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¹ The abbreviations used are: SOR, sulfite:cytochrome *c* oxidoreductase; MPT, molybdopterin; EPR, electron paramagnetic resonance; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; bp, base pairs; BTP, bis-trispropane-HCl buffer; ECF, extracytoplasmic function.

to be a *c*-type cytochrome in all cases studied so far) catalyze the final step in the degradation of sulfur-containing amino acids and have been extensively studied in mammalian and avian systems (1). SORs from these sources are located in the intermembrane space of the mitochondria (2). They form a molybdoenzyme family with assimilatory nitrate reductases and are homodimers (molecular mass, 100–112 kDa) containing one heme *b* and one molybdopterin (MPT)-type pterin molybdenum cofactor/subunit (1). The recently published crystal structure of the chicken liver enzyme revealed the presence of three functional domains: a heme *b*-coordinating domain, an MPT binding domain and a so-called "dimerization domain," which has been assigned a major role in the formation of the homodimeric structure of these enzymes. SORs have also been studied by electron paramagnetic resonance (EPR), resonance Raman, and electron spin echo-envelope modulation spectroscopies (3–5) and exhibit spectroscopic properties distinct from those of other families of molybdenum-containing enzymes. One particularly distinguishing feature in the spectroscopy of the eukaryotic SORs studied to date is the appearance of $I = 1/2$ superhyperfine splitting of the Mo(V) EPR signal at low pH.

In addition to the eukaryotic sulfite:acceptor oxidoreductases, enzymes catalyzing the same reaction have been found in photo- and chemotrophic sulfur-oxidizing bacteria (6–8). During oxidation of reduced sulfur compounds such as thiosulfate or sulfide by obligately or facultatively chemolithotrophic sulfur oxidizers (most of them formerly grouped as *Thiobacilli*), sulfite is formed as a free or bound intermediate of the complex oxidation processes (9, 10). In these organisms sulfite can be oxidized to sulfate either directly by SOR (EC 1.8.2.1), which can occur either as free enzymes or as part of thiosulfate-oxidizing multienzyme complexes (10, 11), or via the energy conserving adenosine-5'-phosphosulfate reductase pathway. The presence of one or both of these enzymes has been established for various *Thiobacilli*, and multienzyme complex-independent sulfite:acceptor oxidoreductases have been enriched or partially purified from various chemotrophic sulfur oxidizers (6). None of these SORs, however, have been purified to homogeneity. Analysis of the available information on these enzymes shows that they form a very heterogeneous group; membrane-bound and soluble SORs can be found as well as enzymes that are inhibited by AMP or stimulated by its addition.

SOR from *Thiobacillus novellus*, a member of the α -proteobacteria, has been described previously as a monomeric, heme, and molybdenum cofactor-containing enzyme with an apparent molecular mass of ~40 kDa (12) that reacts with a cytochrome c_{550} from the same organism (13). However, more

TABLE I
Bacterial strains, plasmids and phage vectors used in this study

Bacterial strain/vectors	Genetic markers/phenotype	References
<i>T. novellus</i> DSMZ 506 ^T	wild type	(69, 70)
<i>E. coli</i> strains		
DH5 α	F Φ 80/ <i>lacZ</i> Δ M15(<i>lacZYA-argF</i>)U169 <i>recA1 endA1 hsdR17</i> (τ K ⁻ mK ⁺) <i>supE44</i> <i>thi-1 gyrA relA1</i>	Life Technologies
XL-1 Blue MRF [']	Δ (<i>mcrA</i>)183 Δ (<i>mcrCB-hsdSMR-mrr</i>)173 <i>endA1 supE44 thi-1</i> <i>recA1 gyrA96 relA1 lac[F'proAB lac^qZ</i> Δ M15 Tn 10 (Tet ^r)]	Stratagene
XL0LR	Δ (<i>mcrA</i>)183 Δ (<i>mcrCB-hsdSMR-mrr</i>)173 <i>endA1 supE44 thi-1</i> <i>recA1 gyrA96 relA1 lac[F'proAB lac^qZ</i> Δ M15 Tn 10 (Tet ^r)] SU- (nonsuppressing) λ^r (lambda resistant)	Stratagene
pGEM-T vector	Amp ^r , <i>lacZ'</i>	Promega
pBK-CMV	Kan ^r , Neo ^r , <i>lacZ'</i>	Stratagene
Lambda Zap Express		Stratagene
pTNSOR2	Amp ^r , <i>lacZ'</i> pGEM-T derivative, <i>T. novellus</i> 300-bp insert, partial <i>sorA-Gen</i>	This study
pTNSOR32	Kan ^r , Neo ^r , <i>lacZ'</i> , pBK-CMV derivative, <i>T. novellus</i> ~8.5- kilobase <i>Sau3AI</i> fragment, <i>sor</i> region	This study
pTNSOR34	Kan ^r , Neo ^r , <i>lacZ'</i> , pBK-CMV derivative, <i>T. novellus</i> ~6.5- kilobase <i>Sau3AI</i> fragment, <i>sor</i> region	This study

TABLE II
Purification of sulfite:cytochrome *c* oxidoreductase from 20 g of frozen *T. novellus* cell material

Cells were grown lithoautotrophically on thiosulfate. For details see text. UC, ultracentrifuge.

	Volume	Total activity	Activity	Protein content	Specific activity	Purification factor	Yield
	ml	units	units/ml	mg/ml	units/mg	-fold	%
Crude extract	57.5	11903	207.0	23.8	8.7	0	100
UC-supernatant	53.0	10319	194.7	16.8	11.6	1.3	87.0
Supernatant 40% (NH ₄) ₂ SO ₄	58.0	9805	169.0	11.5	14.7	1.7	82.4
Supernatant 50 °C heat step	58.0	11187	192.9	11.8	16.4	1.9	94.0
Sediment 70% (NH ₄) ₂ SO ₄	23.0	11318	492.1	25.4	19.4	2.2	95.0
DEAE-Sephacel-pool	62.0	9137	147.4	2.4	62.3	7.2	76.8
HTP-pool, after ultrafiltration	2.8	5219	186.4	11.6	160.4	18.4	43.9
Superdex 75-pool	13.6	4390	322.8	1.3	257.2	29	36.9
Mono Q-pool	4.0	1311	514.9	1.7	327.9	37.7	11

extensive characterization of the type of pterin molybdenum cofactor contained in the enzyme, the exact nature of the heme group, and further properties of this or any other of the *Thiobacillus* SORs remained outstanding. On a molecular biological level, even less data are available: to date the only confirmed gene sequence for a bacterial SOR is the *soxC* gene (14) from *Paracoccus pantotrophus* GB17 (15) (formerly *Thiosphaera pantotropha* (16)), which encodes an enzyme that is part of a multienzyme complex.

The present work describes the first spectroscopic and enzymological characterization of a bacterial member of the sulfite oxidase family, the SOR from *T. novellus*. The relationship of *T. novellus* SOR with other SOR enzymes is discussed based on analysis of its properties and gene sequences.

EXPERIMENTAL PROCEDURES

Materials—Chemicals were purchased from Merck, Sigma, and Fluka in per analysis or corresponding quality.

Bacteria, Media, and Plasmids—Bacteria and plasmids used are listed in Table I. *T. novellus* was cultivated in modified DSMZ medium 69 containing 4.2 g/l Na₂HPO₄, 1.5 g/l KH₂PO₄, 0.3 g/l yeast extract, 0.3 g/l NH₄Cl₂, 0.1 g/l MgSO₄, 10 g/l Na₂SO₃·5 H₂O, 5 ml/l trace element solution, final pH 8.5. The trace element solution was prepared as specified for DSMZ medium 69. MgSO₄ and thiosulfate were autoclaved separately. For solid medium, 1.5% agar were added. 200-ml cultures were aerated by vigorous shaking and incubated for 3–4 days at 30 °C; 10-liter cultures were grown in carboys with forced aeration over 6–7 days. Sterile 1 M Na₂CO₃ was used to maintain a pH of about 8.5. After 2–3 days, 500 ml of a thiosulfate feeding solution (200 g/l) were added to 10-liter cultures every second day. Cells were harvested by centrifugation, and the pellet was stored at –20 °C. *Escherichia coli* strains were grown in liquid or solidified Luria-Bertani, 2 × YT medium or, as required, NZY medium (17).

SOR Purification—Except where otherwise stated, all purification steps were performed at 4 °C. 20 g of frozen *T. novellus* cell material were homogenized in 3 volumes of 10 mM Tris-HCl, pH 7.8, and cells were broken by sonication (1 min/ml) in a Branson sonifier. Cell debris was removed by centrifugation (Sorvall RC-5B, 18 000 × g, 30 min), and the resultant crude extract was subjected to ultracentrifugation (Beckman L5–50; 2 h, 145,000 × g). This was followed by an ammonium sulfate precipitation (45% saturation), centrifugation, and heat treatment of the supernatant (50 °C, 30 min in the presence of 20 mM sodium sulfite). Following quick chilling of the extract and the removal of precipitate by centrifugation, ammonium sulfate was added to 70% saturation (*cf.* Ref. 12), and the resultant precipitate was then dissolved in 10 mM Tris-HCl, pH 7.8, and dialyzed against 2 × 5 liters of the same buffer for ~16 h. The dialyzed extract was applied to a DEAE-Sephacel column (1.6 × 15 cm), equilibrated in the same buffer, and eluted using a linear sodium chloride gradient (400 ml, 0–500 mM NaCl). The sulfite-oxidizing activity eluted between 120 and 180 mM NaCl. Active fractions were pooled, dialyzed extensively against 2 × 5 liters of 1 mM potassium phosphate buffer, pH 7.8, and subsequently applied to a hydroxyapatite column (HTP Biogel, Bio-Rad, 2.6 × 20 cm) buffered in 1 mM potassium phosphate buffer, pH 7.8. A 400-ml linear gradient of 1–50 mM phosphate was applied, and SOR activity eluted between 5 and 20 mM potassium phosphate. Enzyme-containing fractions were selected for pooling so that a maximum separation from a contaminating cytochrome *c*₅₅₁ was achieved. Fractions were concentrated by ultrafiltration (Centriprep-10, Amicon). The retentate was gel-filtered on a Superdex 75 16/60 column (Amersham Pharmacia Biotech) equilibrated with 10 mM Tris-HCl, pH 7.8, containing 150 mM NaCl, and fractions exhibiting SOR activity were desalted and subjected to strong anion exchange chromatography on a MonoQ 5/5 fast protein liquid chromatography column (10 mM Tris-HCl, pH 7.8; gradient, 0–250 mM NaCl in 50 ml). Active fractions, which eluted at NaCl concentrations between 130–200 mM, were checked for purity using SDS-PAGE.

SOR Activity Measurement—SOR activity was determined spectrophotometrically using a Perkin-Elmer Lambda 11 spectrophotometer.

The standard reaction mixture contained 0.05 mM cytochrome *c* from horse heart (Sigma) and a known amount of SOR in 20 mM Tris-HCl, pH 8.0, in a final volume of 1 ml. The reaction was carried out at $23 \pm 1^\circ\text{C}$ and was started by the addition of sulfite (final concentration, 2.5 mM; stock solution, 200 mM in 50 mM Tris-HCl, pH 8.8, 5 mM EDTA). The rate of reaction was quantified by monitoring the increase in absorbance at 550 nm; 1 unit was defined as the amount of enzyme oxidizing 1 μmol of sulfite/min. Extinction coefficients for cytochrome *c* were as given by the manufacturer (cytochrome *c* from horse heart) or as determined by the method of Berry and Trumpower (18) (*T. novellus* cytochrome c_{550}). All activity determinations were taken as the mean of three measurements and were corrected for non-enzymatic sulfite oxidation. Measurements in which cytochrome *c* was supplied in non-saturating concentrations (determination of reaction mechanism) were carried out in the presence of 20 mM EDTA, *i.e.* under partly inhibiting conditions as described in Ref. 19 to overcome detection limits of measurements with a light path of 1 cm. The presence of EDTA in the reaction mix raises the affinity of the enzyme for cytochrome *c* by ~ 1 order of magnitude, while there is no obvious effect on the affinity for sulfite. Assays with non-saturating substrate concentrations and partial inhibition of the enzyme hence contained 20–100 μM cytochrome *c* and 2–16 μM sulfite; assays with only one substrate varied, and the second substrate at saturating concentrations contained 1–12 μM cytochrome *c* and 4–100 μM sulfite. Catalytic constants were determined both from Hanes-Plots and by direct nonlinear fitting (least squares method) using the Scientist software (Micromath), both methods yielding similar results. Data for mixed type inhibition were fitted to the following equation (20).

$$\nu = \frac{V_{\max}}{\left(1 + \frac{I}{K_{iu}}\right) + \left(1 + \frac{K_m}{S}\right)\left(1 + \frac{I}{K_{ic}}\right)} \quad (\text{Eq. 1})$$

The constants K_{ic} and K_{iu} refer to the competitive and the non-competitive component, respectively, of the inhibition pattern observed. Characterization of the mechanisms of reaction and inhibition was performed according to Cleland (21, 22). Statistical errors are given as the standard deviation of the mean.

Denaturing PAGE—SDS-PAGE was carried out according to the method of Laemmli (23). Native PAGE was carried out using essentially the same method but without the inclusion of SDS and denaturing agents. Gels were stained for protein using either Coomassie Brilliant Blue or silver (24). The method of Cohen and Fridovich (25) was employed for SOR activity staining; heme-dependent peroxidase activity was identified on denaturing gels using 3'-3'-diaminobenzidine (26) and on native gels by enhanced chemiluminescence (27).

Purification of *c*-Type Cytochromes from *T. novellus*—Three *c*-type cytochromes were enriched or purified from *T. novellus* as a byproduct of the SOR purification procedure. They were separated from the SOR preparation either after DEAE anion exchange or after hydroxyapatite chromatography (cytochrome c_{551}). Whereas cytochrome c_{550} was purified by subsequent cation exchange chromatography (28), cytochrome c_{552} and c_{551} were further enriched by strong anion exchange chromatography (MonoQ 5/5, Amersham Pharmacia Biotech) and gel filtration on a Superdex 75 16/60 column (Amersham Pharmacia Biotech).

Amino acid sequencing was performed using an Applied Biosystems gas phase sequenator after Western blotting on a polyvinylidene difluoride membrane (Millipore) or tryptic digest and HPLC separation of samples.

Determination of Heme and Mo Content—Heme type and content were determined spectrophotometrically in alkaline pyridine solutions according to the method of Berry and Trumpower (18). Molybdenum content was quantified colorimetrically as described previously (29, 30). Midpoint potentials of heme groups were measured as in Refs. 31 and 32.

Identification of Pterin Molybdenum Cofactor—The molybdenum cofactor was characterized by analytical reverse-phase (2 xHypersil C18, 126×4.6 mm; particle size, 5 μm) HPLC chromatography of oxidation products of the cofactor following liberation from SOR upon denaturation of the enzyme (33–35). Xanthine oxidase (Sigma) and Me_2SO reductase from *Rhodobacter capsulatus* (36) were used as reference sources of MPT and molybdopterin-guanine dinucleotide forms of molybdenum cofactor.

Spectroscopic Measurements—UV-visible absorption spectrophotometric measurements were carried out using either a Shimadzu UV-3000 double beam spectrophotometer or a Perkin-Elmer Lambda 11 spectrophotometer. EPR spectroscopy was carried out at the Center for Magnetic Resonance at the University of Queensland, using a Bruker

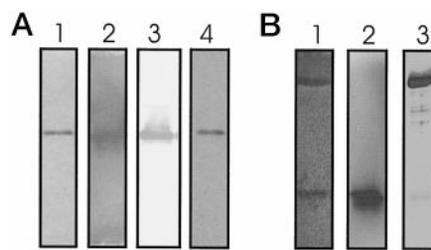


FIG. 1. Detection of purified *T. novellus* SOR in 7.5% native PAGE (A) and 17.5% SDS-PAGE (B). Each lane contained between 0.5 and 3 μg purified protein, depending on the detection method used. A, lane 1, Coomassie Brilliant Blue stain; lane 2, SOR activity stain (25); lane 3, immunostaining after Western blotting; lane 4, heme stain (27). B, lane 1, Coomassie Brilliant Blue stain; lane 2, heme stain (26); lane 3, immunostaining after Western blotting.

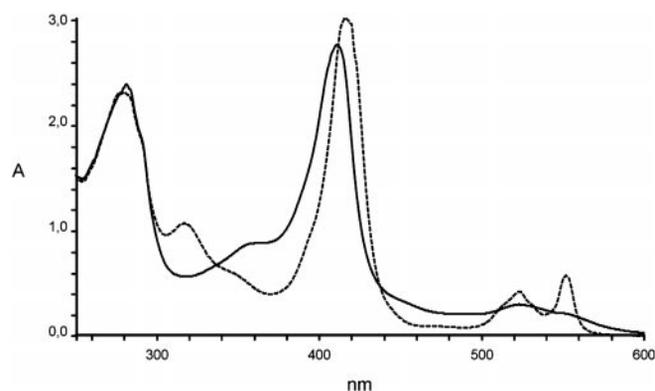


FIG. 2. Optical spectra of purified SOR from *T. novellus*. The spectra were recorded in 10 mM Tris-HCl, pH 7.8, and the reduced sample contained 2 mM sulfite. Solid line, oxidized enzyme; dashed line, sulfite-reduced enzyme.

ESP300E spectrometer equipped with a TE₁₀₂ rectangular resonant cavity and a cold nitrogen flow cryostat. Temperature control was effected using a Eurotherm B-VT2000 variable temperature controller. A microwave power of 10 milliwatts, a modulation frequency of 100 kHz, and a modulation amplitude of 0.25 millitesla were employed; the temperature was kept constant at 130 K. Other signal acquisition parameters were selected so that the spectral resolution was limited by the modulation amplitude. Microwave frequencies between 9.460 and 9.465 GHz were monitored using an EIP 548B microwave counter, and field positions were recorded using a Bruker ER 035M NMR Gaussmeter. Samples for EPR contained 0.4 mg/200 μl of purified enzyme in either 50 mM Tricine, pH 8.8, or HEPES, pH 7.0. To generate the Mo(V) species of SOR, 20 mM sulfite were added. Computer simulations of Mo(V) EPR spectra were carried out as described in earlier work (37).

Immunological Methods—SOR antiserum was prepared in rabbit by injection of 2×750 μg of purified SOR. Western blotting and immunodetection were carried out using standard procedures (17). SOR antiserum was diluted 1:2000.

Molecular Biological Methods—Standard molecular biological techniques were used throughout (17, 38). Restriction enzymes were purchased from Life Technologies, Inc. *Taq* DNA polymerase was from Promega. Genomic DNA from *T. novellus* was isolated as described by Nano and Kaplan (39). A representative genomic library of partially *Sau*3AI-digested *T. novellus* DNA was constructed from fragments >6 kilobases in the lambda ZAP express vector (Stratagene) according to the manufacturer's instructions. DNA sequencing was carried out by Sequiserve (Vaterstetten, Germany) using an automated sequencer and the chain-termination method (40).

Amplification of an SOR Probe and Hybridization Experiments—Degenerate oligonucleotides deduced from the amino acid sequence of N-terminal and tryptic fragments of SorA (TNSOR2, 5'-CG(C/G)CC(C/G)CT(C/G)GT(C/G)ATG TA-3'; TNSOR4, 5'-GC(C/G)AG(C/G)GG(T/C)TC(G/C)AC GAA-3') were used to amplify a 300-bp polymerase chain reaction product (55 $^\circ\text{C}$ annealing, 30 cycles, 1 mM MgCl_2), which was subsequently cloned into pGemT (Promega). SOR probes for use in hybridization experiments were labeled nonradioactively by polymerase chain reaction using digoxigenin-11-dUTP (Roche Pharma-

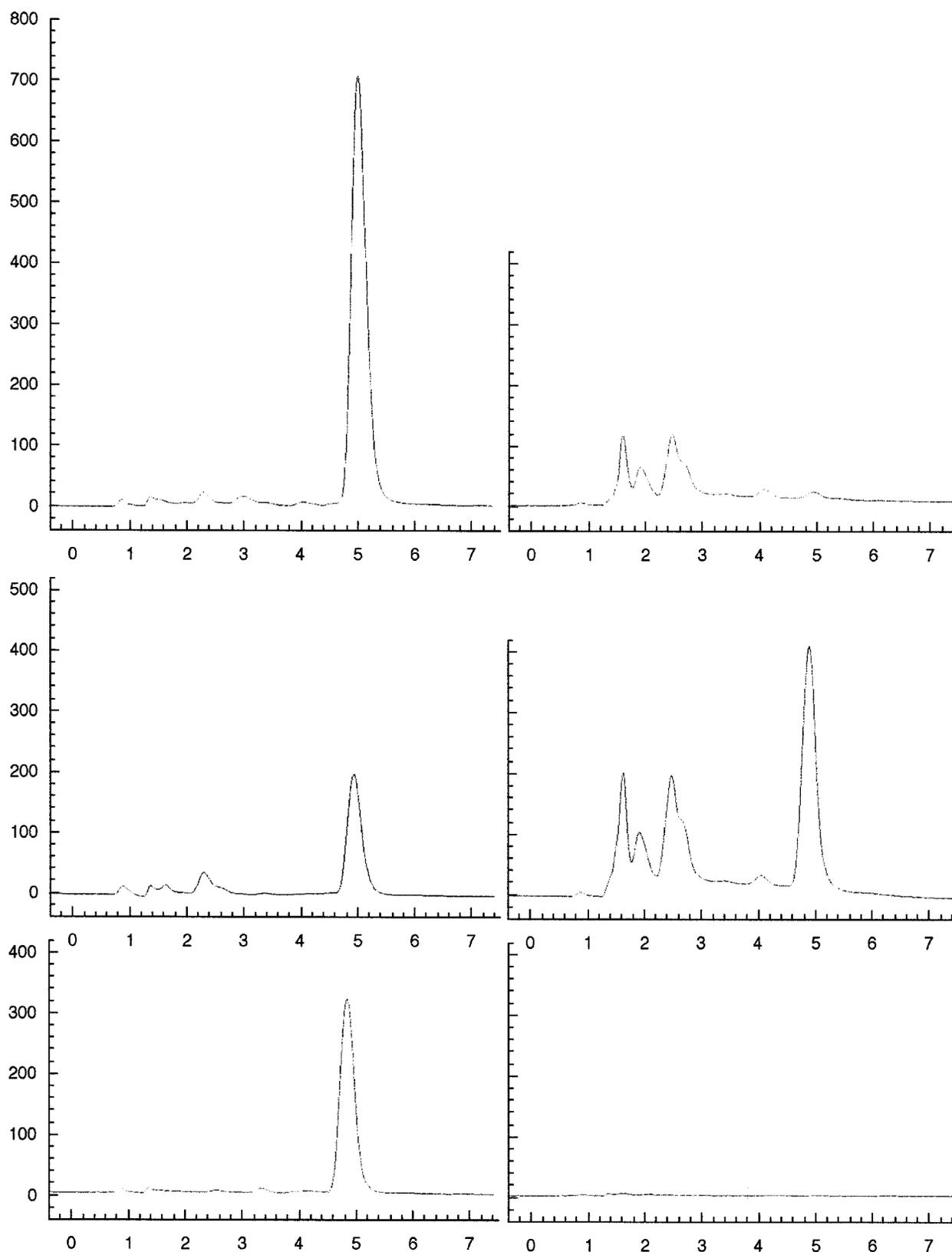


FIG. 3. **Characterization of the pterin molybdenum cofactor of *T. novellus* SOR.** Xanthine oxidase (buttermilk) and Me₂SO reductase (*R. capsulatus*) were used as reference proteins for MPT and molybdopterin-guanine dinucleotide forms of the cofactor. The cofactor was detected as an oxidized derivative (form A) after reverse phase chromatography on HPLC. *Left column*, detection of form A; *right column*, detection of form A dinucleotide. *Top row*, xanthine oxidase; *middle row*, Me₂SO reductase; *bottom row*, *T. novellus* SOR. The elution profile of the cofactor extracted from SOR resembles that of xanthine oxidase, identifying the cofactor as a molybdopterin.

ceuticals). Homologous hybridizations were carried out at 68 °C for 16–20 h.

GenBank™ Accession Numbers and Computer-based Analysis—The

gene sequence of the *sor* region from *T. novellus* has been deposited with GenBank™ under accession number AF154565. Analysis of the *sor* region DNA sequence was undertaken using the WebAngis Software

package (ANGIS), and sequence similarity searches were performed using the BLAST algorithm (41).

RESULTS

Sulfite:cytochrome *c* oxidoreductase from *T. novellus* was purified using a modified and expanded protocol based on the procedure in Ref. 12; the results of the purification are summarized in Table II. The enzyme eluted from the final strong anion exchange column in two overlapping peaks containing the reduced and the oxidized form of SOR, as determined by the UV-visible absorption of the cytochrome component. Native PAGE of the purified SOR revealed the presence of only one protein band upon Coomassie staining (Fig. 1A). This band also stained for SOR activity and cross-reacted with the *T. novellus* SOR antiserum (Fig. 1A). SDS-PAGE of the purified enzyme yielded two Coomassie-staining bands with apparent molecular masses of ~40 and ~8 kDa, respectively, identifying the enzyme as a heterodimer (Fig. 1B). The overall molecular mass of the enzyme as determined after SDS-PAGE is in good agreement with a value of ~46 kDa obtained after gel filtration on Superdex 75.

Characterization and Content of Cofactors—Optical spectra of the purified enzyme clearly reveal the presence of a sulfite-reducible *c*-type heme with the following absorption maxima in the reduced state (values in parentheses refer to maxima in the oxidized spectrum): α , 552 nm (–); β , 523 nm (–); γ , 416 nm (411 nm); δ , 317 nm (363 nm) (Fig. 2). Heme staining after native and denaturing PAGE identified the heme as an integral part of the enzyme (Fig. 1A) with the heme group being covalently bound by the 8-kDa component of the holoenzyme (Fig. 1B). Absorption spectra of purified SOR in alkaline pyridine confirmed that the heme group contained in *T. novellus* SOR is a *c*-type heme (18). SOR contains 0.8 ± 0.15 heme groups/holoenzyme as estimated from the absorption spectrum. Redox titration at pH 8.0, the pH at which SOR exhibited maximum activity, suggested a midpoint potential for this heme group of $E_{m8.0} = +280$ mV.

Colorimetric determination of Mo content gave an average value of 1.2 ± 0.25 Mo atoms/holoenzyme. The analytical data then suggest a content of one molybdenum atom and one heme group/ $\alpha\beta$ -holoenzyme. The type of pterin molybdenum cofactor contained in SOR was elucidated by liberation and subsequent oxidation of the cofactor followed by reverse phase HPLC chromatographic analysis. As there are several known forms of the pterin molybdenum cofactor, xanthine oxidase (Sigma) and dimethyl-sulfoxide reductase from *R. capsulatus* (36) were used as reference sources of MPT and molybdopterin-guanine dinucleotide-type cofactors, respectively. The dinucleotide form of the pterin molybdenum cofactor is often found in bacterial enzymes (3). The elution profile of the cofactor oxidation products from the reverse phase HPLC column clearly shows that SOR from *T. novellus* contains an MPT-type cofactor (Fig. 3), as the oxidized cofactor extracted from this enzyme shows the same elution profile as that from xanthine oxidase but is distinct from that exhibited by Me_2SO reductase.

EPR Spectroscopy—Although samples of the resting enzyme in Hepes, pH 7.0 (Fig. 4A), and in Tricine, pH 8.8 (Fig. 4A), did not give rise to a Mo(V) EPR signal, upon the addition of 20 mM sulfite to either sample a well defined Mo(V) EPR signal was observed (Fig. 4A). The *g*-values, anisotropy, narrow line widths, and particularly the weak features at 350 and 355 millitesla justify the assignment of the signal to Mo(V); the spacings and intensities of these latter features are consistent with their being due to the ~25% natural abundance of $I = 5/2$ $^{95/97}\text{Mo}$ nuclei. The spectra were simulated (Fig. 4A) assuming g_1 , 1.9914; g_2 , 1.9661; and g_3 , 1.9541. A high resolution spectrum of the g_1 -signal recorded at a lower modulation am-

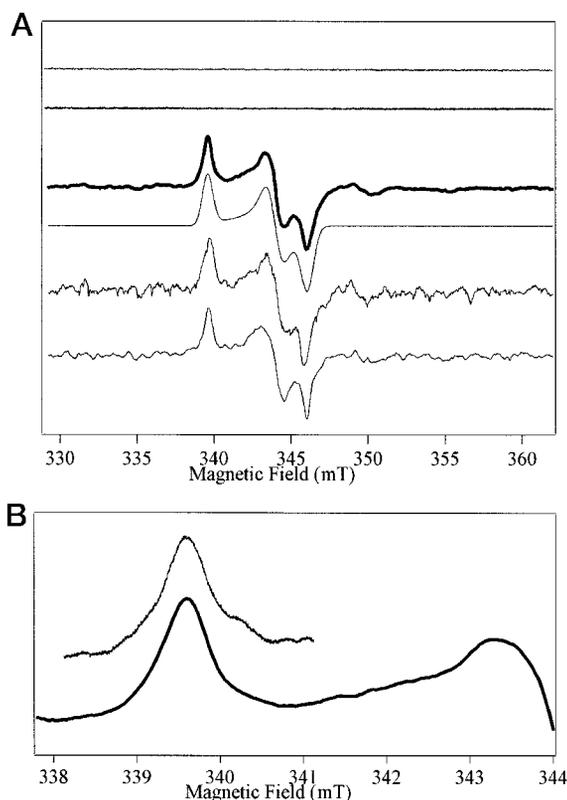


FIG. 4. EPR-spectra of *T. novellus* SOR. A (from top to bottom), resting enzyme in 20 mM Tricine, pH 8.8; resting enzyme in 20 mM Hepes, pH 7.0; sulfite-reduced enzyme in Tricine, pH 8.8; simulation of sulfite-reduced enzyme in Tricine assuming g_1 , 1.9914; g_2 , 1.9661; and g_3 , 1.9541; sulfite-reduced enzyme in Hepes, pH 7.0, containing 200 mM NaCl; sulfite-reduced enzyme in 0.5 M phosphate buffer, pH 7.0, containing 200 mM NaCl. B, high resolution spectrum of sulfite-reduced enzyme in Hepes, pH 7.0, containing 200 mM NaCl; reproduction of Fig. 4A sulfite-reduced enzyme in Tricine on the same scale as Fig. 4B (high resolution spectrum of sulfite-reduced enzyme in Hepes, pH 7.0). Spectra were recorded at 130 K, 10 milliwatt microwave power, 0.25 or 0.1 millitesla modulation amplitude, 100 kHz modulation frequency. Spectra are presented normalized in intensity for enzyme concentration; between 10 and 80 spectra were averaged in each case.

plitude (0.1 millitesla; Fig. 4B) clearly showed that the “wings” present on this feature are asymmetrically positioned about the main $I = 0$ g_1 -feature and are thus due to underlying $^{95/97}\text{Mo}$ hyperfine lines rather than to either unresolved super-hyperfine splitting or to $\Delta m_I = 1$ ^1H “spin-flip” transitions. Studies at up to 200 milliwatt microwave power (not shown) also provided no evidence for ^1H spin-flip transitions, though at 130 K these may be expected to be difficult to detect (*cf.* Ref. 42).

At both pH values the signals obtained were very similar to the so-called “high pH” EPR signal that can be readily observed with avian and mammalian sulfite oxidoreductases (43–45). However, another characteristic EPR signal of these enzymes, the “low pH” form, was not observed with *T. novellus* SOR samples at pH 7.0 and could not be induced by the addition of 200 mM NaCl (Fig. 4A), as reported for the eukaryotic enzymes (43, 44). Within the limits of the noise level of the data, the signal from *T. novellus* SOR at pH 7.0 and containing 200 mM Cl^- (Fig. 4A) is indistinguishable from that seen at high pH (Fig. 4A). The EPR spectrum (Fig. 4A) of a sulfite-reduced SOR sample in 0.5 M phosphate buffer, pH 7.0, containing 200 mM NaCl was also similar to the one seen at high pH. Thus, the difference in behavior of the *T. novellus* enzyme and the eukaryotic enzyme is not merely an effect of Hepes buffer anion binding. The signal in phosphate buffer is, perhaps, slightly

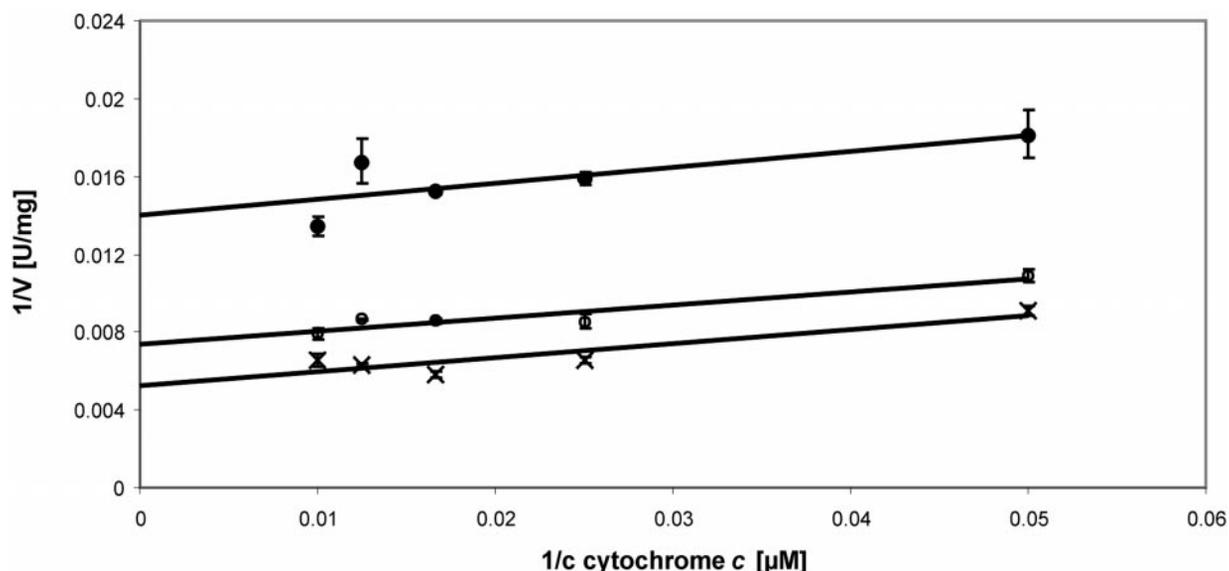


FIG. 5. **Determination of the reaction mechanism of sulfite:cytochrome *c* oxidoreductase.** Double reciprocal plot of data obtained by activity measurements using partially inhibited enzyme (19) and both substrates, sulfite and cytochrome *c*, in non-saturating concentrations for the conditions used. The data plotted show a variation of cytochrome *c* concentration in the assay mixture at several constant sulfite concentrations. The set of parallel lines obtained suggests the presence of a ping-pong mechanism. Sulfite concentrations: ●, 4 μM sulfite; ○ 8 μM sulfite, × 12 μM sulfite. Error bars are given as the standard deviation of the mean.

different in that the g_2 -feature appears to be asymmetrically broadened, the g_3 -feature appears to have a lower line width, and a shoulder is apparent on the high field side of this feature. Nevertheless, the best simulation of this signal as a single species was still that of Fig. 4A. The samples investigated failed to elicit the characteristic phosphate-inhibited signal (45, 46) observed in eukaryotic sulfite oxidoreductases under similar conditions. It would appear that the ability of the molybdenum center to adopt a low pH ligand environment is an obligatory prerequisite for subsequent formation of the phosphate-inhibited species and hence this latter species is also not observed in the *T. novellus* enzyme.

Enzymological Characterization of SOR—The pH optimum of the reaction catalyzed by SOR from *T. novellus* was determined in 20 mM Tris-HCl and 20 mM bis-trispropane-HCl (BTP) buffer in steps of 0.5 pH units and was found to be pH 8.0 and pH 8.5, respectively. Apart from the slightly higher optimum, the reaction showed a much greater pH dependence in BTP buffer than in Tris, with the activity falling to 65% of the maximum within 1 pH unit either side of pH 8.5 in BTP, whereas the corresponding values in Tris-HCl were 80 and 95% of the maximum (data not shown). The optimum temperature of the SOR-catalyzed reaction was determined to be 65 °C, about 35 °C above the optimum growth temperature of *T. novellus*. Preincubation of oxidized and sulfite-reduced SOR solutions at various temperatures with subsequent determination of the remaining activity confirmed the great heat stability of this enzyme; although samples of oxidized SOR lost 50% activity after incubation at 60 °C for 2 min, 50% inactivation of the reduced enzyme was only observed after 15 min at 70 °C (data not shown).

An isoelectric point of pH 5.5 was determined by preparative isoelectric focusing on a MonoP column (Amersham Pharmacia Biotech). Controlled tryptic digestion of *T. novellus* SOR had no influence on enzyme activity as had already been reported (12) and as would be expected for a heterodimeric protein. N-terminal sequencing of both subunits and tryptic digestion of the larger, Mo-containing subunit yielded the amino acid sequences indicated in Fig. 6.

Catalytic Properties—Apparent K_m values for sulfite and cytochrome *c* (horse heart) at saturating concentrations of the

TABLE III
Inhibition of SOR activity by buffer substances, salts, and the substrate analogue nitrate.
Activity measurements were carried out using the standard assay, and buffered as specified in the table.

Compound	50% inhibition of SOR activity
Tris-HCl, pH 8.0	120 mM
bis-Trispropane-HCl, pH 8.0	40 mM
Potassium-phosphate buffer, pH 8.0	20 mM
EDTA (in 20 mM Tris-HCl, pH 8.0)	10 mM
NaCl (in 20 mM Tris-HCl, pH 8.0)	50 mM
Potassium-nitrate (in 20 mM Tris-HCl, pH 8.0)	3 mM

second substrate were determined to be 27 and 4 μM , respectively. To investigate the reaction mechanism, measurements of enzyme activity with both substrates supplied in nonsaturating concentrations were carried out. A double reciprocal plot of the data (Fig. 5) yielded a set of parallel lines, suggesting the presence of a ping-pong mechanism (47). This kind of mechanism is also found in the eukaryotic SORs and in this case involves two active centers. V_{max} as determined from a secondary plot was 2500 units/mg. The calculated V_{max} is only valid under the experimental conditions used.

Inhibition Studies—SOR from *T. novellus* is strongly inhibited by anions and buffer substances such as phosphate and bis-trispropane as well as by the substrate analogue nitrate (Table III). The enzyme is subject to product inhibition by sulfate, which shows a mixed-type non-competitive pattern with respect to sulfite. K_{ic} and K_{iu} values were determined to be 24 and 16 mM for sulfite. A similar product inhibition pattern with a K_{ic} value of 8 mM for sulfite (determined from a Dixon-Plot) has been reported for the chicken liver SOR (19).

Reactivity of SOR with Purified *c*-Type Cytochromes—Three *c*-type cytochromes were purified or enriched from *T. novellus* extracts. These heme-containing proteins have apparent molecular masses of 12.6 kDa (c_{550}), 75 kDa (c_{551}), and 125 kDa (c_{552}), with purity coefficients ($E_{\text{soret red}}/E_{280}$) of 5.65, 2.79, and 0.996. Purified preparations of the *c*-type cytochromes were used as electron acceptors in SOR activity assays. It was found that SOR reacts exclusively with cytochrome c_{550} , as previously proposed (13). This finding is consistent with the midpoint

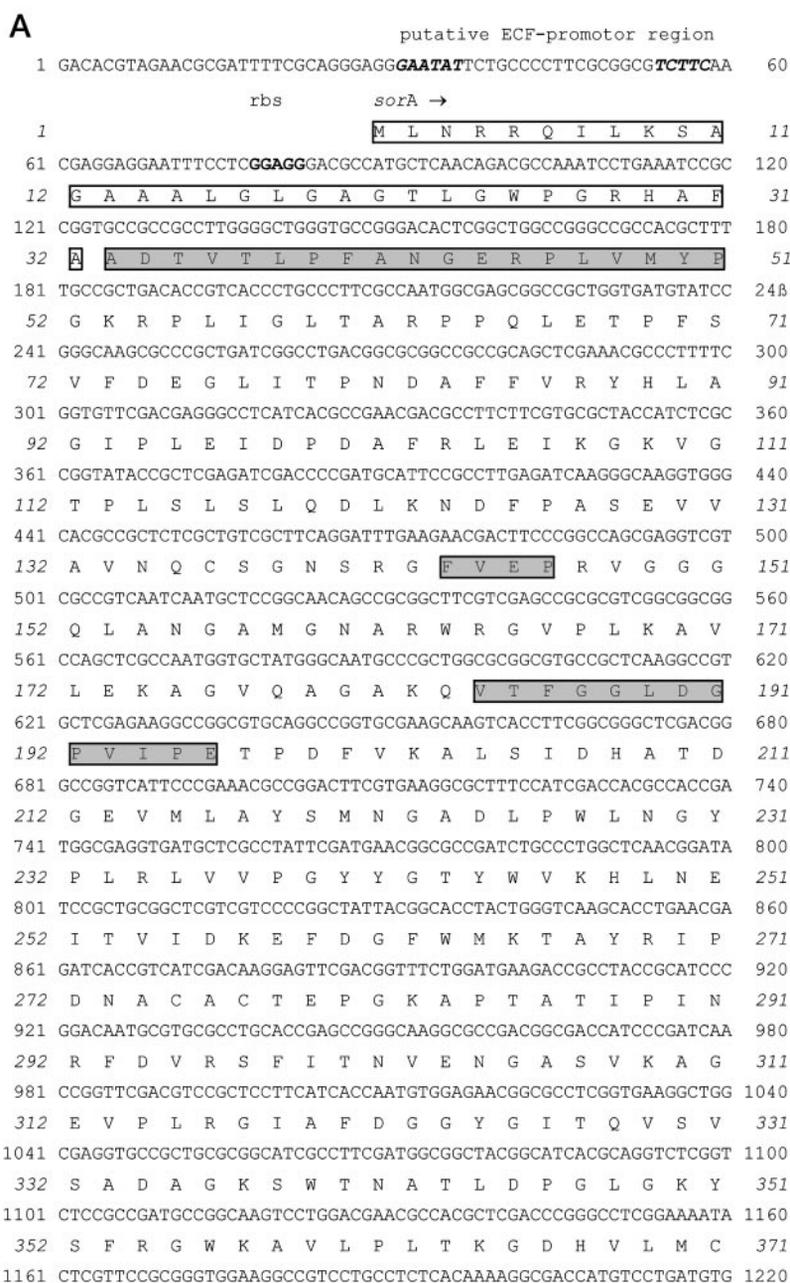


FIG. 6. Sequence of the gene region containing *sorA* and *sorB*, the structural genes of *T. novellus* SOR. The presence of both a putative promoter and terminator region suggests that these genes form an operon. *sorAB* encode the amino acid sequences determined for the subunits of the purified protein. *Open boxes*, leader peptides; *gray boxes*, N-terminal and tryptic amino acid sequences determined for the subunits of *T. novellus* SOR; *rbs*, ribosome binding site; *arrows*, position of hairpin loop structure (free energy of formation, -109.2 kJ/mol). GenBank™ accession No. AF154565. *Numbers* refer to the bp numbering, *numbers* in *italics* refer to the amino acid numbering of the encoded proteins.

potentials at pH 8.0 determined for cytochromes c_{550} , c_{551} , and c_{552} , which were $+302$, $+253$, and $+242$ mV. The apparent K_m value of SOR for the native cytochrome c_{550} under substrate-saturating conditions was $2.5 \mu\text{M}$, a value almost identical to the one determined for the artificial acceptor cytochrome from horse heart. Furthermore, this value is sufficiently low as to be possibly physiologically relevant.

Cloning and Analysis of the *sor* Region from *T. novellus*—Two-hybrid phages, $\lambda\text{TNSOR32}$ and $\lambda\text{TNSOR34}$, containing the *sor* gene region were isolated from a representative genomic library in lambda ZAP express using a partial *sorA* gene probe. The probe was amplified from genomic DNA using degenerate oligonucleotides deduced from the N-terminal and tryptic amino acid sequences of SorA, the large SOR subunit. After excision of the phagemid vectors pTNSOR32 and pTNSOR34 from the hybrid phages, 2378 bp of the insert of pTNSOR32 (Fig. 6) were sequenced on both strands by primer walking.

Analysis of the *sorAB* Gene Sequence—The sequenced region

(Fig. 6) contains two open reading frames encoding a 40.15-kDa (43.4 kDa unprocessed) polypeptide with strong similarities to known SORs (error probabilities: eukaryotic SORs, between 5×10^{-39} and 8×10^{-35} , bacterial SoxC protein 1×10^{-25}) and a novel 8.8 kDa (11.55 kDa unprocessed) mono-heme *c*-type cytochrome. The two open reading frames, designated *sorA* and *sorB*, are separated by only 16 bp and encode the amino acid sequences determined for the subunits of the purified SOR (Fig. 6). Putative ribosome binding sites were identified for both open reading frames (Fig. 6). A putative promoter for ECF-type sigma factor-dependent expression (48) is located upstream of *sorA* and a possible hairpin loop terminator structure with a calculated free energy of formation of -109.2 kJ/mol is found 122 bp downstream of *sorAB*, suggesting that *sorAB* form an operon. The proposed promoter region, which contains a -35 region “GAATAT” followed by -10 region “TCTTC” at a distance of 17 bp, retains 64% of the suggested consensus sequence for ECF-dependent promoters (48), which consists of a -35 region “GAACTT” and a -10 region “TCTRA”

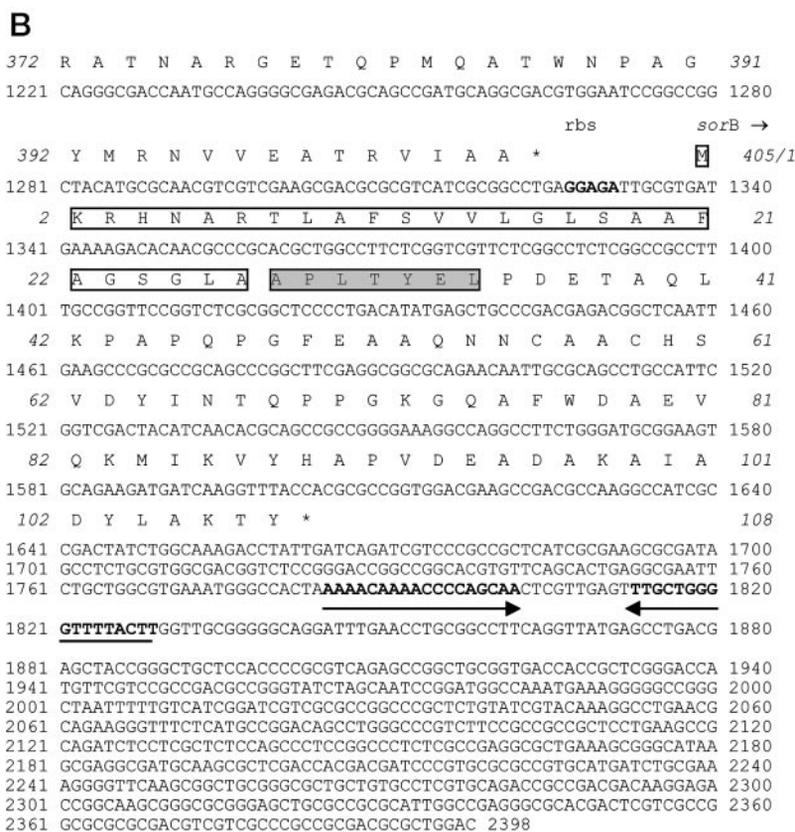


FIG. 6—continued

separated by 16–17 bp.

The identification of a putative promoter for ECF-type sigma factor-dependent expression upstream of *sorA* is particularly interesting as ECF-type sigma factors are proposed to regulate genes encoding proteins with extracytoplasmic functions (48). Also, SorA and SorB are preceded by signal sequences. Both observations are indicative of a periplasmic location of the SOR holoenzyme. While SorA is preceded by a 32-amino acid leader of the double arginine type (49), which is likely to target the protein for export via the *tat* system (50, 51), the SorB signal sequence (27 amino acids) is characteristic of *Sec*-dependent export (52). The similarity of the *sorA* gene product to eukaryotic SORs is restricted to two core domains, the molybdopterin binding and the dimerization domain. No similarities between either SorA or SorB and the heme *b* binding domain of the eukaryotic enzymes were detected. A conserved cysteine, which coordinates the MPT cofactor in the eukaryotic enzymes (53), is also found in SorA (Fig. 7). This finding is perhaps to be expected from the similarity of the EPR signal of *T. novellus* SOR to the high pH signal of the eukaryotic enzymes.

The *sorA* and *sorB* gene products are soluble proteins; there is no suggestion that SOR may be a transmembrane or membrane-associated protein. The calculated pI values of 6.52 and 4.67 for the processed SorA and SorB polypeptides, respectively, are consistent with the experimentally determined value pI = 5.5 for the holoenzyme.

DISCUSSION

The characterization described herein of the sulfite:cytochrome *c* oxidoreductase from *T. novellus* has revealed a novel, heterodimeric type of SOR. The previously described 40-kDa protein (12) corresponds to the pterin molybdenum cofactor binding subunit of the enzyme. The unidentified heme moiety described in the earlier work on the enzyme (12) has been found to reside on a separate, 8.8-kDa subunit, which has been shown by sequence analyses, alkaline pyridine denaturation and elec-

tronic absorption spectroscopy to be a *c*-type cytochrome with a novel polypeptide primary sequence. Hence, the structure of this enzyme is clearly distinct from that of the well studied, eukaryotic SORs, which are homodimeric proteins with a heme *b* group and a pterin molybdenum cofactor residing on each of the identical subunits.

Similar to the eukaryotic SORs, the *T. novellus* SOR contains an MPT-type cofactor. This is somewhat unusual, because most of the bacterial molybdoenzymes (3, 54) characterized to date contain a dinucleotide form of the pterin molybdenum cofactor. At the same time, most of these enzymes are members of the Me₂SO reductase family, a notable exception being the molybdopterin-cytosine dinucleotide-containing aldehyde oxidase from *Desulfovibrio gigas* (55). The discovery of an MPT-type cofactor in a bacterial enzyme belonging to the sulfite oxidase family and in a bacterial member of the third family of molybdoenzymes, the xanthine oxidase family (56), does, however, suggest some specificity of distribution of the forms of the pterin molybdenum cofactor among the molybdoenzyme families.

T. novellus SOR appears to be located in the bacterial periplasm as is indicated by the presence of sequences encoding signal peptides in the gene sequences of both subunits. This observation is supported by the fact that the subunits of the mature protein are both N-terminally processed. Given that eukaryotic SORs are found in the mitochondrial intermembrane space (2), all SORs studied so far seem to reside in cell compartments outside the cytoplasm and have a similar topological position with respect to the respiratory chain. Inspection of the leader sequences encoded by *sorA* and *sorB* implies that translocation of the two subunits to the periplasm likely occurs by two distinct pathways. The MPT binding SorA subunit is exported via the *tat* system (50, 51), known to export proteins that are at least partially folded and with the cofactors inserted, whereas SorB is targeted for *Sec*-dependent export.

FIG. 7—continued

suoxcaen	VAYHMNGVDI	PRDHGAPLRV	IVPGNVGARQ	VKWLKRIIVS	EKESDSHWQQ	
pdsoxc	LAFFFANGEAL	RKEHGYPARL	VVPGWEGNMW	VKVVRRLLGIY	DKA...VES	
suoxchi	LAYEMNGTEL	PRDHRFPVRV	VVPGVVGAR	VKWLRRVAVS	PDESFSRWQQ	
suoxrat	LAYEMNGQPL	PRDHGFPVRV	VVPGVVGARH	VKWLGRVSV	SEESYSHWQR	
suoxhum	LAYEMNGQPL	PRDHGFPVRV	VVPGVVGARH	VKWLGRVSVQ	PEESYSHWQR	
tnsor	LAYSMNGADL	PWLNQYPLRL	VVPGYYGTYW	VKHLNEITVI	DKEFDGFWMK	
○ ○ ○ ○						
dimerization domain						
suoxcaen	KDYR	AFSPA	V NIG.DELKWN	TVPSIQEYPV	QCAICSPAPN	TKVDRGDET
pdsoxc	R..E	ETSKYT	DL...MPDGR	ARKWTWVMDA	KSVITSPSPQ	VPIRHGKGPL
suoxchi	NDYK	GFSPCV	DW..DTVDFR	TAPAIQELPV	QSAVTQPRPG	AAVFPGE..L
suoxrat	RDYK	GFSPSV	DW..DTVDFD	LAPSIQELPI	QSAITQPQDG	TTVESGE..V
suoxhum	RDYK	GFSPSV	DW..DTVDFD	SAPSIQELPV	QSAITEPRDG	ETVESGE..V
tnsor	TAYR	IPDNAC	ACTEPGKAPT	ATIPINRFDV	RSFITNVENG	ASVKAGE..V
○						
suoxcaen	DISGYAWS	GG	GRGIIRIEVS	VDGGETWNSV	EMEQUEEKQDQ	EHMYAWTLFK
pdsoxc	VISGLAWS	GN	GR.ITRVDVS	LDGGKNWTTA	RITGQ..ALP	K...ALTRFH
suoxchi	TVKGYAWS	GG	GREVVVDVS	LDGGRTWKVA	RLMGDK.APP	GRAWAWALWE
suoxrat	IIKGYAWS	GG	GRAVIRVDVS	MDGGLTWQEA	ELEGEE.QHP	RKAWAWRIWQ
suoxhum	TIKGYAWS	GG	GRAVIRVDVS	LDGGLTWQVA	KLDGEE.QRP	RKAWAWRLWQ
tnsor	PLRGIAFDGG	YG.ITQVSVS	ADAGKSWTNA	TLDPG...LG	K..YSFRGWK	
○						
suoxcaen	AEVKIPPGVK	EFNIIAKAVD	RAYNTQETA	SGIWNVRG..	.LIHN...A	
pdsoxc	LDIDWDGS..	EMLLQSRVD	ETGYVQ.TK	DALRAIRGRN	NVYHNNGIQT	
suoxchi	LTVPVEAG.T	ELEIVCKAVD	SSYNVQFDSV	APIWNLRG..	.VLST...A	
suoxrat	LKAHPAEQK	ELNIIKAVD	DSYNVQDTV	APIWNLRG..	.VLSN...A	
suoxhum	LKAPVPAGQK	ELNIVCKAVD	DGYNVQDTV	APIWNLRG..	.VLSN...A	
tnsor	AVLPLTKG..	DHVLNCRATN	ARGETQ..M	QATWNPAG..	.YMRN...V	
○						
suoxcaen	WHR....VP	IIVKD				
pdsoxc	WVVKADGEVE	NVEIA				
suoxchi	WHR.....				
suoxrat	WHR....VH	VQVVP				
suoxhum	WHR....VH	VYVSP				
tnsor	VEA....TR	VIAA.				

oxidation of SO_3^- to SO_4^{2-} is a two-electron process, whereas the electron storage capability of the enzyme is three electrons (two on the Mo ion as $\text{Mo(VI)} \rightarrow \text{Mo(IV)}$ and one on the heme group as $\text{Fe(III)} \rightarrow \text{Fe(II)}$). As electron egress occurs via the heme group, which can only store one electron, two reactions with a one-electron acceptor are required to restore the enzyme to the Mo(VI) -containing state whereby another sulfite anion can be oxidized. We were able to show that SOR from *T. novellus* does indeed catalyze the oxidation of sulfite by a ping-pong type mechanism, with the stable intermediate form characteristic of ping-pong mechanisms being the sulfite-reduced enzyme ($t_{1/2} > 48$ h). In conjunction with the physical data obtained for the enzyme our results suggest that, unlike the hybrid ping-pong mechanism discovered in Ref. 59, which is a combination of a two site ping-pong mechanism with a random ternary mechanism, SORs combine a two site ping-pong mechanism with a (probably random) sequential mechanism at the second, the heme site. The sulfite oxidation reaction catalyzed by SOR is also sensitive to high concentrations of various anions and buffer substances (19, 60, and this study), and the pH optimum of the reaction, as well as the overall pattern of activity, are influenced by the type of buffer employed. The strong inhibition of *T. novellus* SOR by nitrate may be caused by formation of a catalytically inactive enzyme-nitrate complex.

In view of the different domain structures of the eukaryotic and the bacterial enzymes it is interesting that both types show enhanced heat stability when reduced with sulfite. In eukaryotic enzymes this property has been attributed to a change in position of the heme *b* group relative to the main enzyme body (4, 61), since this domain is connected to the rest of the enzyme

via an exposed hinge region of amino acids. This adjustment of the position of the heme group is thought to facilitate intramolecular electron transfer processes (61). The observation made suggests that despite the fact that the catalytic groups of the bacterial SOR reside on different, noncovalently connected subunits, a reduction of this enzyme by sulfite also results in intramolecular changes reflected in the altered physical properties reported.

Despite the overall similarity of the two types of sulfite: cytochrome *c* oxidoreductase the results of EPR spectroscopy suggest that the active site at the molybdenum center of *T. novellus* SOR might be different from that of the eukaryotic enzymes. Samples investigated failed to elicit a pH-dependent change in EPR signal, which was hitherto thought to be characteristic of this type of enzyme. These findings are in contrast to those of Toghrol and Southerland (12) who reported a pH-dependent change of EPR signal conformation in Tris-buffered samples. The low pH form obtained by these authors, however, appears to be a mixed species of high and low pH signal. It seems likely that the coordination sphere of the molybdenum atom in the bacterial enzyme does not allow for the change in position of the Mo(V)-OH species thought to be responsible for the change between the high and low pH form of the chicken liver enzyme (5). Hepes buffer is known to bind strongly to the molybdenum centers of some enzymes and thus we attempted to generate the low pH signal in phosphate buffer. However, not only did the low pH signal remain elusive, we were also unable to generate the phosphate-inhibited EPR signal observable in the eukaryotic enzyme. Again, these findings imply a lack of coordination flexibility in *T. novellus* SOR compared with the eukaryotic enzymes. Activity measurements suggest

that this apparent lack of conformational flexibility of at least the Mo(V) state does not preclude catalytic activity at a level comparable to the eukaryotic enzymes.

T. novellus is one of the facultatively autotrophic members of the formerly so-called "Thiobacilli" and exhibits a great versatility as far as utilization of substrates is concerned. In 1997, Kelly *et al.* (10) suggested that the oxidation of sulfur compounds in this organism might proceed via the so-called "Paracoccus pathway," which involves a multienzyme-complex and is found in *Paracoccus versutus* (formerly *T. versutus* (62)) and *P. pantotrophus* (15). One reason for this assumption may have been the close phylogenetic relation between these members of the α -proteobacteria. Analysis of the *sor* gene region, however, suggests that oxidation of sulfur compounds via SOR does not involve a multienzyme complex. The SOR structural genes *sorAB* appear to form an independent operon with a putative promoter for ECF-type sigma factor-dependent expression upstream of *sorA* and a terminator structure downstream of *sorB*. This structure does not resemble the environment of the *soxC* gene from *P. pantotrophus*, which is located in a genetic region containing the other genes relevant for the function of the thiosulfate-oxidizing multienzyme complex (11, 14). SOR expression in *T. novellus* is induced by the presence of thiosulfate in the growth medium (63), and SOR can amount to about 1.6% of the total cell protein in cells grown lithoautotrophically with thiosulfate, suggesting a major role for the thiosulfate oxidation pathway in which SOR catalyzes the final step, the conversion of the oxidation intermediate sulfite to sulfate.

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