Purification of Thiobacillus novellus Sulfite Oxidase

EVIDENCE FOR THE PRESENCE OF HEME AND MOLYBDENUM*

Freshette Toghril and William M. Southerland†
With the technical assistance of Nathaniel Hughes
From the Department of Biochemistry and Cancer Center, Howard University College of Medicine, Washington, D.C. 20059

Sulfite oxidase from *Thiobacillus novellus* has been purified 206-fold. The enzyme reduced both ferricyanide and cytochrome c. The ferricyanide activity was 3–5% of the cytochrome c activity. During purification, the absorbance ratio of *A*135 nm/*A*280 nm showed a continual increase, suggesting the presence of heme in the *T. novellus* sulfite oxidase molecule. The absorption spectrum of the enzyme is very similar to that of rat liver sulfite oxidase which contains cytochrome b₅ type heme. Gel electrophoresis of the purified protein in the presence of sodium dodecyl sulfate revealed a protein staining band of approximately 41,000 Da. These results suggest that *T. novellus* sulfite oxidase is a monomeric protein. EPR spectroscopy of the enzyme revealed a characteristic molybdenum spectrum, which was observed only in the presence of sulfite. Analysis of the *T. novellus* sulfite oxidase molybdenum cofactor showed a fluorescence spectrum indistinguishable from that displayed by the molybdenum cofactor of chicken liver sulfite oxidase. Therefore, it is concluded that *T. novellus* sulfite oxidase is a monomeric (M₀ ~ 40,000) molybdenohemoprotein.

Sulfite oxidase from mammalian and avian liver is a molybdenohemoprotein with two identical subunits. Each subunit contains one molybdenum and one heme (1–7). The enzyme uses cytochrome c as its physiological electron acceptor and is also capable of reducing ferricyanide (8, 9). Proteolytic treatment of rat liver sulfite oxidase abolishes cytochrome c reduction and results in the separation of intact molybdenum- and heme-containing domains. These results provided evidence that the sulfite oxidase domains are connected by an accessible hinge peptide (6, 7).

In contrast to mammalian and avian liver sulfite oxidases, heme has not been reported in sulfite oxidases from bacterial sources (10–12). The enzyme from *Thiobacillus thioparus* contains 1 mol of non-heme iron/M₀ = 54,000 (10), while the enzyme from *Thiobacillus ferroxoxidans* (M₀ = 41,500) contains neither heme nor non-heme iron (11). *Thiobacillus novellus* sulfite oxidase has also been reported as a non-heme protein (12). Finally, Kessler and Rajagopalan (5) have reported the presence of a sulfite-dependent molybdenum EPR signal in partially purified *T. thioparus* sulfite oxidase. Results presented here provide evidence for the presence of heme and molybdenum in *T. novellus* sulfite oxidase.

MATERIALS AND METHODS

*T. novellus* (ATCC 50073) was grown in the modified autotrophic media of Charles and Suzuki (13) containing 4.0 g of KH₂PO₄, 1.5 g of KH₂PO₄, 10 g of Na₂SO₄, 5.4 g of CaCl₂-2H₂O, 0.02 g of FeCl₃·6H₂O, 0.1 g MgSO₄·7H₂O, 0.5 g of (NH₄)₂SO₄, 0.02 g of MnSO₄·2H₂O, 1.1 g of NaHCO₃, 4 × 10⁻³ M (NH₄)₂MoO₇·4H₂O, 10⁻⁴ M biotin, 0.2 ml of 0.2% phenol red in a total volume of 1 liter. Media (500 ml or less) were autoclaved at 110 °C for 20 min. Twelve-liter carboys containing 6–8 liters of media were sterilized at 110 °C for 60 min.

Stock cultures of cells were kept on solid agar autotrophic media and were transferred to fresh media every 4 weeks. Media volumes of 500 ml or less were inoculated with one loop of cells obtained from solid cultures. Larger volumes were inoculated with 1 volume of a liquid autotrophic culture/12 volumes of culture medium. The cells were grown in a controlled temperature room at 30 °C with vigorous stirring or forced aeration. The pH of cultures were maintained at 7.5 by the addition of 5% Na₂CO₃ through a 0.22-μm Millipore filter. After 6 days, cultures were checked for purity, harvested, washed with 1 mM potassium phosphate, pH 7.8, and stored at −20 °C until used.

Horse heart cytochrome c (Type XI), DEAE-cellulose, and biotin were from Sigma. All inorganic chemicals were obtained from Mallinckrodt Chemical Works. Aff-Gel blue and hydroxylapatite were from Bio-Rad.

A Sorvall refrigerated centrifuge (RC5B) was used for all centrifugations. Cells were sonicated using a Virsion cell disrupter model 16500, and pH measurements were obtained using a Beckman model 3500 digital pH meter. Conductivity measurements were performed using a Wescan conductivity meter. Ultraviolet and visible spectra of the enzyme were recorded using a Beckman DU-8 spectrophotometer. The effect of heat on the stability of oxidized and reduced sulfite oxidase was determined using a temperature-controlled GCA model 25 shaking water bath. EPR studies were performed using a Varian E9 HF spectrophotometer at frequency modulation of 100 kHz. Sample temperature was −140 °C. HPLC gel chromatography of the purified enzyme was achieved using an LDC-3 Constametric pump, a Spectrophysics model 8440 variable wavelength detector, and a TSK-SW 3000 size exclusion column.

Sulfite oxidase activity was measured as described by Johnson and Rajagopalan (4). Generally, enzyme assay mixtures contained 0.04 mM cytochrome c, 0.4 mM sodium sulfite, and an aliquot of enzyme in 0.1 M Tris-HCl, pH 8.5, to a final volume of 2.5 ml. In assays measuring ferricyanide reduction, cytochrome c was replaced with 2 mM potassium ferricyanide. In all cases the reaction was started by addition of sodium sulfite. The enzyme assays were performed at 25 °C also using a Beckman DU-8 spectrophotometer. One unit of sulfite oxidase activity was defined as the amount of enzyme which caused

---

*This work was supported by Grant GM27975 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom reprint requests should be addressed.

1 The abbreviation used is: HPLC, high pressure liquid chromatography.
an absorbance change of 1.0/min. Protein concentrations were determined according to the procedure of Bradford (14). Electrophoresis in the presence of sodium dodecyl sulfate was performed according to the procedures of Laemmli (15), Studier (16), and Vesterberg (17). Molybdenum cofactor analyses were performed as described by Johnson et al. (18).

Molecular weight of T. novellus sulfite oxidase in an aqueous buffer was determined by gel chromatography using Ultrogel AcA 34 at 4 °C in 0.2 M potassium phosphate, pH 7.8. Column dimensions were 3 x 103 cm. Molecular weight standards used were aldolase (158,000), rat liver sulfite oxidase (120,000), ovalbumin (45,000), chymotrypsinogen A (25,000), and ribonuclease A (13,700). Blue dextran was used as an internal volume marker. Protein-containing fractions collected off the Ultrogel column were monitored for absorbance at 280 nm to indicate the void volume of the column, and methylene blue was used as an absorbance change of 1.0/min.

RESULTS

The washed cells were suspended in 5 volumes of 1 mM potassium phosphate buffer, pH 7.8, and sonicated under nitrogen at 4 °C for a total of 30 min with 1-min cooling intervals every minute. The sonicate was then centrifuged at 43,500 x g for 30 min (all subsequent centrifugations were also for 30 min at 43,500 x g). The resulting supernatant was made 40% saturated with ammonium sulfate by the addition of the solid salt. The ammonium sulfate solution was centrifuged, and sodium sulfite was added to the supernatant to a final concentration of 0.01 M. The solution was then heated at 58 °C for 30 min. After heating, the mixture was centrifuged. The supernatant was treated with ammonium sulfate to 70% saturation and kept at 4 °C for 20 min and centrifuged again.

The 70% ammonium sulfate precipitate was dissolved in 1 mM potassium phosphate buffer, pH 7.8, and dialyzed for 3–4 h against a large volume of the same buffer with a single buffer change. When the conductivity of the dialyzed enzyme solution reached that of 5 mM potassium phosphate or less, the material was chromatographed on a small column (2 x 10 cm) of DEAE-cellulose. Prior to the elution of the enzyme, the column was washed thoroughly with a large volume of the starting buffer. The enzyme was subsequently eluted with a linear potassium phosphate gradient (5–50 mM). The sulfite oxidase activity eluted between 20 and 30 mM phosphate. Fractions containing the highest sulfite oxidase activity was subsequently pooled and concentrated by ultrafiltration. The conductivity of the concentrated enzyme solution was adjusted to that of 5 mM potassium phosphate buffer by the addition of 1 mM potassium phosphate buffer. The resulting enzyme solution was next applied to a column of hydroxylapatite (2.5 x 20 cm) which had been equilibrated at 4 °C with 5 mM potassium phosphate, pH 7.8, and eluted with a gradient of 5–50 mM phosphate buffer. The pooled fractions

| Step                  | Vol | Total activity | Activity | Protein | Specific activity | Purification | Yield |
|-----------------------|-----|----------------|----------|---------|------------------|--------------|-------|
|                       | ml  | units          | units/ml | mg/ml   | units/mg protein | fold         | %     |
| 1. Sonicate x g supernatant | 150 | 21,150         | 146      | 5.36    | 26.3             | 1            | 100   |
| 2. 43,500 x g supernatant | 135 | 22,680         | 168      | 2.43    | 68               | 1.5          | 100   |
| 3. 40% (NH₄)₂SO₄ | 143 | 15,015         | 105      | 1.83    | 57.3             | 2.1          | 70.2  |
| 4. Heat step | 158 | 20,838         | 151      | 1.51    | 100              | 3.5          | 98.5  |
| 5. 40–70% (NH₄)₂SO₄ | 150 | 18,900         | 126      | 1.20    | 105              | 3.9          | 89.0  |
| 6. DEAE-cellulose | 43  | 11,180         | 259      | 0.25    | 1,052            | 40           | 52.8  |
| 7. Hydroxylapatite | 10  | 3,820          | 382      | 0.09    | 4,063            | 154          | 18.0  |
| 8. Affi-Gel blue | 10  | 3,820          | 382      | 0.07    | 5,418            | 206          | 18.0  |
off hydroxylapatite were applied to an Affi-Gel blue column which had also been equilibrated with 5 mM potassium phosphate. The enzyme was not absorbed to the column, and the fractions containing sulfite oxidase activity were collected and subsequently concentrated. The 413/280 nm ratio of the preparation continually increased with purification. At steps 5, 6, and 7, the 413/280 nm ratios of the enzyme preparation were 0.66, 0.96, and 1.6, respectively. This observation initially suggested the presence of heme in the enzyme. The results of the T. novellus sulfite oxidase purification procedure described above are summarized in Table I. Sulfite oxidase activity was measured both with ferricyanide and cytochrome c as electron acceptors in all steps of the purification procedure. The ferricyanide reductase activity was stable at 3-5% of the cytochrome c activity. Both electron acceptors showed maximum activity at pH 8.5. The enzyme was purified 206-fold with respect to its cytochrome c activity.

The absorption spectrum of T. novellus sulfite oxidase is shown in Fig. 1 and is very similar to the spectrum of mammalian sulfite oxidases, which contains cytochrome b5 type heme (1-6). Fig. 2 shows the visible absorption spectra of oxidized and dithionite-reduced T. novellus sulfite oxidase. The oxidized sample displays a γ peak at 412 nm. This peak in the reduced sample is shifted slightly in the direction of longer wavelengths and its absorbance is increased by approximately 50%. Also present in the spectrum of the reduced sample is an α peak at 550 nm and a β peak at 521 nm.

Polyacrylamide gel electrophoresis of the enzyme in the presence of sodium dodecyl sulfate showed only one band which exhibited a molecular weight of approximately 40,000 (Fig. 3). Gel chromatography of the enzyme on Ultrogel AcA 34 in aqueous buffer also revealed a molecular weight of the enzyme close to 40,000 (Fig. 4). Consequently, it was concluded that T. novellus sulfite oxidase exists in solution as a monomer. HPLC gel chromatography of purified T. novellus sulfite oxidase showed that a single elution peak was observed when absorbance at 280 or 413 nm was monitored. Furthermore, the elution positions of material absorbing at 413 nm and material absorbing at 280 nm totally coincided (Fig. 5). These observations provided clear evidence for the presence of heme in the structure of T. novellus sulfite oxidase. HPLC chromatography also confirmed that the size of the bacterial enzyme was significantly smaller than the enzyme from chicken liver.

EPR spectroscopy of purified T. novellus sulfite oxidase was performed in order to unequivocally identify molybdenum as part of the enzyme structure. EPR analysis revealed molybdenum spectra in the presence of sulfite (Fig. 6) which responded to pH changes in a manner similar to what had been observed for rat liver sulfite oxidase (19). These results provided decisive evidence for the presence of active molybdenum in the T. novellus enzyme.

The molybdenum components of mammalian and avian sulfite oxidases exist in the proteins as part of a cofactor. The molybdenum cofactor has been prepared and characterized by Johnson et al. (18). The isolated cofactor displayed fluorescence spectra very similar to that observed for pterin. Moreover, the cofactor was found to have pterin in its structure. Analysis of T. novellus sulfite oxidase cofactor is shown in Fig. 7. As can be seen, the fluorescence spectrum of the

Fig. 3. Sodium dodecyl sulfate gel electrophoresis of T. novellus sulfite oxidase on 7.5% polyacrylamide gels. Gel a contains molecular weight standards (ovalbumin, 43,000; α-chymotrypsinogen, 25,700; and β-lactoglobulin, 18,000). Gel b contains purified T. novellus sulfite oxidase.

Fig. 4. Determination of the aqueous molecular weight of T. novellus sulfite oxidase by chromatography on Ultragel AcA 34. ○, elution positions of molecular weight standards; △, elution position of T. novellus sulfite oxidase.
Purification of T. novellus Sulfite Oxidase

FIG. 5. HPLC gel chromatography of T. novellus and chicken liver sulfite oxidases. Trace A shows elution of T. novellus sulfite oxidase (solid line corresponds to 280 nm absorbance; broken line corresponds to 413 nm absorbance). Trace B shows the elution of chicken liver sulfite oxidase. Absorbance at 413 nm was monitored. Flow rate was 1 ml/min. Buffer used was 0.25 M potassium phosphate, pH 7.8.

FIG. 6. Molybdenum EPR spectra of T. novellus sulfite oxidase at pH 7.0 and 9.0 in 0.1 M Tris-HCl buffer.

FIG. 7. Fluorescence spectra of the molybdenum cofactor from T. novellus sulfite oxidase (---) and chicken liver sulfite oxidase (---).

FIG. 8. Heat stability of oxidized and reduced T. novellus sulfite oxidase at 58 °C. Equal concentrations of oxidized (---) and reduced (---) enzyme were incubated at 58 °C in a shaking water bath for 60 min. Aliquots were removed at regular time intervals to determine percent SO₃⁻ to cytochrome c activity remaining. Protein concentrations were 4.2 mg/ml of 40-70% ammonium sulfate-fractionated enzyme. The reduced enzyme sample was 5 mM in sulfite, while the oxidized sample contained an equal concentration of sulfate.

Rat liver sulfite oxidase consists of two distinct molybdenum- and heme-containing domains joined by a flexible hinge peptide (16). The domains may be separated using limited proteolysis which results in loss of the cytochrome c activity. The rat liver enzyme displays increased domain interaction in the reduced state as evidenced by increased heat stability over that of the reduced molybdenum domain (20). Proteolytic treatment of T. novellus sulfite oxidase with trypsin at room temperature for up to 5 h at trypsin to enzyme ratios of 1/20 and 1/1 did not affect the activity of the enzyme. Exposure of the oxidized bacterial enzyme to heat (58 °C) resulted in rapid inactivation. However, the substrate-reduced T. novellus sulfite oxidase remained active for more than 1 h at 58 °C, suggesting increased stabilization of the reduced enzyme, as has been observed with the enzyme from rat liver (Fig. 8).

T. thiopeus contains both an AMP-dependent system capable of sulfite oxidation, adenosine-5'-phosphosulfate reductase, and an AMP-independent sulfite oxidase (10). Consequently, it was of interest to investigate the influence of AMP on the T. novellus sulfite oxidase activity. Table II shows that AMP does not affect the T. novellus enzyme activity. These results suggested that the T. novellus activity was due to direct catalysis of sulfite oxidation and not to the APS-reductase system. The T. novellus enzyme was significantly inhibited...
To yield the final concentrations shown, and T. novellus sulfite oxidase activity was determined.

| Additions | ΔAbsorbancy |
|-----------|-------------|
| None (control) | 0.168 |
| 2 mM AMP | 0.156 |
| 4 mM AMP | 0.156 |
| 2 mM NaN | 0.048 |
| 4 mM NaN | 0.013 |

by sodium cyanide as has been observed for mammalian sulfite oxidases (3).

**DISCUSSION**

Sulfite oxidase from *T. novellus* has been purified to homogeneity. Since gel electrophoresis in the presence of sodium dodecyl sulfate and gel chromatography in aqueous media gave similar molecular weights for the enzyme (41,000 and 38,000, respectively), it was concluded that *T. novellus* sulfite oxidase exists in solution as a monomer of \( M_\text{r} \sim 40,000 \).

The \( A_{413\text{ nm}}/A_{390\text{ nm}} \) ratio of the enzyme preparation continually increased during purification. In addition, analysis of the visible absorption spectra of the isolated enzyme and the co-elution of 413 and 280 nm absorbing material during HPLC gel chromatography revealed the presence of the heme in the structure of *T. novellus* sulfite oxidase. The bacterial enzyme also exhibited a single band on nondenaturing gels which stained both for heme and protein. Results presented here are in contrast to earlier investigations (12) which reported that *T. novellus* sulfite oxidase did not contain heme.

The EPR spectra of the enzyme was obtained to determine the presence of molybdenum as an active component of *T. novellus* sulfite oxidase. Moreover, a comparison of the fluorescence spectra of the molybdenum cofactor from *T. novellus* and chicken liver sulfite oxidases revealed identical spectra and suggested that the molybdenum cofactor is apparently conserved in sulfite oxidases from evolutionarily diverse sources.

The reduced sulfite oxidase from *T. novellus* was not inactivated by heating at 58 °C, while the oxidized enzyme showed rapid inactivation. The increased heat stability of reduced rat liver sulfite oxidase has been attributed to stronger ligand binding by the molybdenum and to domain interactions in the reduced form of the enzyme (20).

Similarly, the increased heat stability of reduced *T. novellus* sulfite oxidase may also be due to stronger ligand binding of the molybdenum component. The contribution of domain interactions to the heat stability of the *T. novellus* enzyme cannot be adequately assessed at this time, since the domain structure of *T. novellus* sulfite oxidase is still under investigation.

The inability of AMP to influence sulfite oxidation by our preparation provided conclusive evidence that the purified enzyme did not function according to the mechanisms of the adenosine-5′-phosphosulfate reductase sulfite oxidizing system present in *T. thioparus* (21). These results further emphasize that sulfite oxidase isolated from *T. novellus* catalyzes the direct oxidation of sulfite to sulfate.

**REFERENCES**

1. Cohen, H. J., and Fridovich, I. (1971) *J. Biol. Chem.* **246**, 359–366
2. Cohen, H. J., and Fridovich, I. (1971) *J. Biol. Chem.* **246**, 367–373
3. Cohen, H. J., Fridovich, I., and Rajagopalan, K. V. (1971) *J. Biol. Chem.* **246**, 374–382
4. Johnson, J. L., and Rajagopalan, K. V. (1976) *J. Clin. Invest.* **58**, 543–550
5. Keseler, D. L., and Rajagopalan, K. V. (1972) *J. Biol. Chem.* **247**, 6566–6573
6. Johnson, J. L., and Rajagopalan, K. V. (1977) *J. Biol. Chem.* **252**, 2017–2025
7. Southerland, W. M., Winge, D. R., and Rajagopalan, R. V. (1978) *J. Biol. Chem.* **253**, 8747–8752
8. Cohen, H. J., Betcher-Lange, S., Kessler, D. L., and Rajagopalan, K. V. (1972) *J. Biol. Chem.* **247**, 7759–7766
9. Ohshima, N., and Chance, B. (1975) *Arch. Biochem. Biophys.* **170**, 514–528
10. Lyric, R. M., and Suzuki, I. (1970) *Can. J. Biochem.* **48**, 334–343
11. Vestal, J. R., and Lundgren, D. C. (1971) *Can. J. Biochem.* **49**, 1124–1130
12. Charles, A. M., and Suzuki, I. (1966) *Biochim. Biophys. Acta* **128**, 522–534
13. Charles, A. M., Suzuki, I. (1966) *Biochim. Biophys. Acta* **128**, 510–521
14. Bradford, M. (1976) *Anal. Biochem.* **72**, 248–254
15. Laemmli, U. K. (1970) *Nature (Lond.)* **227**, 680–685
16. Studier, F. W. (1973) *J. Mol. Biol.* **79**, 237–248
17. Vesterberg, O. (1971) *Biochim. Biophys. Acta* **243**, 345–348
18. Johnson, J. L., Hainline, E., and Rajagopalan, K. V. (1980) *J. Biol. Chem.* **255**, 1783–1786
19. Johnson, J. L., and Rajagopalan, K. V. (1976) *J. Biol. Chem.* **251**, 5505–5511
20. Southerland, W. M., and Rajagopalan, K. V. (1978) *J. Biol. Chem.* **253**, 8753–8758
21. Lyric, R. M., and Suzuki, I. (1970) *Can. J. Biochem.* **48**, 334–354

Acknowledgments—We thank Dr. K. V. Rajagopalan for the use of his laboratory facilities in the performance of the HPLC and molybdenum cofactor analyses and EPR studies and Dr. J. L. Johnson for assistance in the performance of molybdenum cofactor analyses and EPR studies. We also thank Mary L. Smith for typing the manuscript.
Purification of Thiobacillus novellus sulfite oxidase. Evidence for the presence of heme and molybdenum.
F Toghrol and W M Southerland

J. Biol. Chem. 1983, 258:6762-6766.

Access the most updated version of this article at http://www.jbc.org/content/258/11/6762

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/258/11/6762.full.html#ref-list-1