Molecular Basis of the Biological Function of Molybdenum

MOLYBDENUM-FREE SULFITE OXIDASE FROM LIVERS OF TUNGSTEN-TREATED RATS*

(Received for publication, February 19, 1974)

JEAN L. JOHNSON,† HARVEY J. COHEN, AND K. V. RAJAGOPALAN§

From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710, and Division of Pharmacology and Toxicology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709

SUMMARY

Livers of tungsten-treated rats have been shown to contain inactive protein which cross-reacts with antibody prepared against native rat liver sulfite oxidase. The cross-reacting material (CRM) was shown to be immunologically identical to the native enzyme by Ouchterlony double immunodiffusion. Difference spectrophotometry, acrylamide gel electrophoresis, and immunological analysis of the proteins of the mitochondrial intermembranous space from livers of control and tungsten-treated rats showed that, like native sulfite oxidase, the CRM was localized in this compartment. CRM was quantitated in terms of the amount of the \( b_1 \)-type heme of sulfite oxidase which was precipitable by the antibody and was shown to be present in amounts corresponding to almost 70% of control. Sulfite oxidase CRM was purified by the procedure established for native enzyme and shown to contain the \( b_1 \) heme in stoichiometric amounts and no molybdenum. Tungsten was quantitated by a sensitive colorimetric technique and was found to be incorporated into 35% of the molybdenum-free molecules. Tungsten-containing sulfite oxidase was shown to have no detectable cytochrome \( c \) reductase activity. Tungsten in the enzyme could be visualized by EPR spectroscopy after reduction by dithionite. The signal at \( g = 1.87 \) was very similar in line shape to the \( g = 1.97 \) signal of molybdenum (V) in native sulfite oxidase. The native and inactive proteins were indistinguishable on acrylamide gels in the presence or absence of sodium dodecyl sulfate. The absorption spectra of the two proteins were quite similar in both the oxidized and reduced states, although the conditions required for reduction of the heme chromophore differed. The native enzyme was reduced by sulfite within 1 min; the inactive protein showed 30 to 40% reduction in the course of 30 min in the presence of sulfite, with full reduction achieved only upon the addition of dithionite. The difference spectrum of oxidized native sulfite oxidase minus inactive protein revealed subtle differences in the environments of the heme prosthetic group, hydrophobic aromatic residues, and possibly sulfhydryl-

molybdenum linkages. Activity assays of the native enzyme complexed with antibody showed loss of ability to reduce \( O_2 \) and cytochrome \( c \) but normal ferricyanide activity. EPR studies revealed that the molybdenum center of the enzyme in the complex was rapidly reducible by sulfite and maintained its sensitivity to anionic and pH effects. Sulfite oxidase in other rat tissues was shown to be antigenically similar to the liver enzyme.

Sulfite oxidase and xanthine oxidase are the major molybdenum-containing enzymes which have been identified in the rat. We have previously reported (1) that the administration of 100 ppm of tungsten to rats maintained on a low molybdenum diet results in a dose- and time-dependent loss of the activities of both of these enzymes. Experiments which will be documented in this and the following article have shown that the tissues of these animals contain considerable amounts of inactive, immunologically cross-reacting materials (CRM) of both enzymes. The characterization of hepatic sulfite oxidase CRM as the de-molybdo protein of the enzyme and the purification and properties of this apoenzyme are reported in this paper. The nature of xanthine oxidase CRM is described in the companion article (2).

EXPERIMENTAL PROCEDURE

Male Sprague-Dawley rats were obtained from Hilltop Laboratories and housed in plastic cages. A normal protein diet with the inclusion of 0.7 g of sodium tungstate per kg (409 ppm of tungsten) was obtained from General Biochemicals and administered in conjunction with deionized drinking water to produce sulfite oxidase deficiency. Animals were maintained on this regimen for periods of several months for the studies reported below. Control animals received Purina rat chow and tap water.

Sulfite oxidation in the presence of various electron acceptors was measured as described previously (3). Activity measurements were performed on a Gilford model 2000 recording spectrophotometer. Absorption spectra were recorded on an Aminco-Chance DW-2 or a Cary 14 spectrophotometer. EPR spectroscopy was performed on samples at \(-100^\circ \) with a Varian E-9 HF spectrometer at a modulation frequency of 100 kHz.

The abbreviations used are: CRM, cross-reacting material; IM space, mitochondrial intermembranous space.

* This work was supported in part by Grant GM 00091 from the National Institutes of Health.
† Supported by Predoctoral Traineeship GM 00233 from the National Institutes of Health.
§ To whom reprint requests should be addressed.
Protein was measured by the method of Lowry et al. (4) with bovine serum albumin as standard.

Preparation of Antiserum—Sulfite oxidase was purified from control rat livers as described previously (5). Rabbits were injected with 1 to 2 mg of the protein emulsified with Freund's complete adjuvant (Difco). Antiserum of high titer was maintained by periodic injection with enzyme and incomplete adjuvant. Serum was separated from clotted red cells by centrifugation, and the γ-globulin fraction was partially purified by ammonium sulfate fractionation.

Metal Analyses—Molybdenum was quantitated using a Perkin-Elmer model 107 atomic absorption spectrometer fitted with a heated graphite atomizer (HGA model 2000). Purified enzyme preparations could be ashed directly in the graphite rod of the instrument. Antibody complexes were pre-ashed at 450° in a muffle furnace and the residue dissolved in 4 N HCl. The colorimetric procedure described below was also used.

Tungsten was measured by a modification of the colorimetric procedure of Bickford et al. (6). With this technique it is possible to detect as low as 0.1 μg of tungsten by complexing the metal with tolune-3,4-dithiol (Eastman Kodak) and extracting the complex into butyl acetate for spectrophotometric assay. As shown in Fig. 1, the tungsten-dithiol complex has a characteristic spectrum in the visible range with maximum absorbance between 630 and 640 nm. Molybdenum also complexes with the dithiol reagent; however, as seen in the figure, the absorption spectrum of this complex clearly differs from that of the tungsten complex. It is possible, as suggested by Bickford et al. (6), to assay both metals simultaneously in duplicate samples by adding citrate to one to inhibit formation of the tungsten complex. Samples used for tungsten analysis in the experiments described below, however, were fortuitously extremely low in molybdenum content. Thus, tungsten could be quantitated directly with the simple precaution of verifying that the color obtained was entirely of a tungsten-dithiol complex. It is however, as seen in the figure, the absorption spectrum of this complex clearly differs from that of the tungsten complex. It is possible, as suggested by Bickford et al. (6), to assay both metals simultaneously in duplicate samples by adding citrate to one to inhibit formation of the tungsten complex. Samples used for tungsten analysis in the experiments described below, however, were fortuitously extremely low in molybdenum content. Thus, tungsten could be quantitated directly with the simple precaution of verifying that the color obtained was entirely of a tungsten-dithiol complex with no spectral perturbations imposed by the presence of molybdenum.

Preparation of Mitochondrial Intermembranous Space Proteins—Livers were homogenized in 10 volumes of 0.25 M sucrose using a Thomas Tissue grinder. Mitochondria were obtained by differential centrifugation as follows. The homogenate was spun at 5000 g for 10 min to remove nuclei and cell debris. The nuclear fraction was washed once with 5 volumes of 0.25 M sucrose. The supernatant and wash were combined and centrifuged at 100,000 X g for 1 hr. The resulting pellet was resuspended in a 0.01 M potassium phosphate buffer, pH 7.8, containing 0.1 mM EDTA. The homogenates were centrifuged at 35,000 g for 15 min and the resulting supernatant was used for Ouchterlony double immunodiffusion. As shown in Fig. 2, the supernatant from control rat liver reacts with antibody prepared against purified rat liver sulfite oxidase to give a single precipitin band indicating the presence of only one antigenic species in the rat liver preparation. Moreover, the extract from livers of tungsten-treated rats forms a precipitin band continuous with the control demonstrating the presence of immunologically identical CRM in these livers.

Identification of Sulfite Oxidase CRM in Livers of Tungsten-Treated Rats—Livers from control and tungsten-treated rats were homogenized in 5 volumes of 0.01 M potassium phosphate buffer, pH 7.8, containing 0.1 mM EDTA. The homogenates were centrifuged at 35,000 X g for 15 min and the resulting supernatants spun at 100,000 X g for 1 hr. The high speed supernatants were concentrated 10-fold in an Amicon ultracentrifuge and used for Ouchterlony double immunodiffusion. As shown in Fig. 2, the supernatant from control rat liver reacted with antibody prepared against purified rat liver sulfite oxidase to give a single precipitin band indicating the presence of only one antigenic species in the rat liver preparation. Moreover, the extract from livers of tungsten-treated rats forms a precipitin band continuous with the control demonstrating the presence of immunologically identical CRM in these livers.

Intracellular Localization of Sulfite Oxidase CRM—With use of the antibody preparation it was possible to determine the intracellular localization of sulfite oxidase CRM in the livers of tungsten-treated rats. In livers of control animals the enzyme has been shown to reside in the IM space (13). IM space proteins were prepared as described under "Experimental Procedure," from livers of control and tungsten-treated rats. Residual active sulfite oxidase in the tungsten-treated animals was less than 3% of the control. Fractionation of the 100,000 X g control supernatant with ammonium sulfate removes any contaminating hemoglobin and yields a mixture of proteins, several of which contain heme, but with sulfite oxidase as the predominant if not the only cytochrome. This is evident in the reduced minus oxidized difference absorption spectrum shown in Fig. 3. The spectrum obtained upon reduction by sulfite is
FIG. 2. Ouchterlony double immunodiffusion of liver extracts from control and tungsten-treated rats. Supernatants were prepared from livers as described in the text. Wells 1, 3, and 4, control; Wells 5 and 6, tungsten-treated; center well, antibody prepared against purified control rat liver sulfite oxidase. After formation of precipitin bands the plates were soaked in 0.9% NaCl to remove uncomplexed proteins, dried, and stained with amido black.

altered slightly by the addition of dithionite, due to spectral alterations of other IM space components, but is nevertheless very similar to the reduced minus oxidized difference spectrum of purified native sulfite oxidase. Similar treatment of the IM space preparation from tungsten-treated rats demonstrates the presence of a cytochrome with identical spectral properties but reducible only by dithionite. In order to determine whether this cytochrome is indeed the inactive CRM noted on Ouchterlony plates, an antibody protection experiment was designed.

Equal aliquots of IM space preparation from control rat liver were incubated with varying amounts of antiserum for 20 min at room temperature, and in each case the extent of inhibition of sulfite → cytochrome c reductase activity was compared with that obtained with antibody which had been preincubated with an equal volume of the analogous preparation from livers of tungsten-treated rats. As seen in Fig. 4, preincubation of the antiserum with the IM space from tungsten-treated animals attenuates its ability to inhibit activity in the control preparation. This clearly demonstrates the existence of significant amounts of inactive CRM in the IM space of livers from tungsten-treated rats. Moreover, the fact that the enzyme in the control preparation is fully protected by an equal volume of inactive preparation over a wide range of antibody concentrations suggests that the inactive CRM has an affinity for the antibody which is comparable to that of native enzyme.

Finally, the acrylamide gel pattern of the CRM-containing IM space preparation (Fig. 5) shows the presence of a heme band which does not stain for sulfite oxidase activity but which migrates to the same position as native enzyme. Incubation of the IM space preparation with antibody, followed by centrifugation, specifically removes this heme band.

Quantitation of Sulfite Oxidase CRM—From the experiments described above it was apparent that the inactive sulfite oxidase CRM contained a heme prosthetic group which was spectrally similar to that of native enzyme but which was not reducible by $\text{SO}_3^{2-}$. It was possible to obtain an estimate of the amount of CRM present in livers of tungsten-treated rats by quantitation of the heme content of immune precipitates. The results of such a determination are presented in Table I. Livers from control and tungsten-treated rats were homogenized in 4 volumes of 0.01 M potassium phosphate buffer, pH 7.8, containing 0.1 mM EDTA. The homogenates were centrifuged at 35,000 $\times$ g for 20 min, and the resulting supernatants were spun at 100,000 $\times$ g for 1 hour. Aliquots of the high speed supernatants were incubated with an excess amount of partially purified antiserum for at least 3 hours at 4°. The resulting immune complexes were pelleted by centrifugation at 35,000 $\times$ g for 10 min, and the pink precipitates were washed twice with 10-ml aliquots of cold 0.9% NaCl and once with 10 ml of deionized water. The heme content of the complexes was then determined. As seen in Table I, livers from two tungsten-treated rats which contained essentially no active sulfite oxidase had CRM corresponding to almost 70% of controls. Thus, tungsten treatment, while drastically lowering the activity of sulfite oxidase, has relatively little effect on the steady state level of hepatic sulfite oxidase protein.

Purification of Sulfite Oxidase CRM—Purification of sulfite...
the mitochondrial intermembranous space. Experimental conditions are described in the text. 

1. intermembranous space preparation from control rat liver, containing 1.78 units of sulfite oxidase in 100 μl, incubated with varying amounts of immune serum; 

2. same conditions but with antiserum previously incubated with 100 μl (0.02 unit) of intermembranous space preparation from livers of tungsten-treated rats.


Fig. 4. Demonstration of sulfite oxidase CRM localization in the mitochondrial intermembranous space. Experimental conditions are described in the text. 

- An intermembranous space preparation from control rat liver, containing 1.78 units of sulfite oxidase in 100 μl, incubated with varying amounts of immune serum; 

- Same conditions but with antiserum previously incubated with 100 μl (0.02 unit) of intermembranous space preparation from livers of tungsten-treated rats.


Fig. 5. Polyacrylamide gel electrophoresis of mitochondrial intermembranous space preparations from livers of control and tungsten-treated rats. 

A and B, control preparations (containing 70 units per ml of sulfite oxidase) stained for activity and heme, respectively. 

C, a preparation from a tungsten-treated rat (with about 2% of the control activity) stained for heme. 

D, E, and F, corresponding gels representing the same solutions which have been diluted 1:1 with antiserum, incubated for 20 min at room temperature, and centrifuged at 15,000 × g for 10 min to remove antigen-antibody complexes which were formed. The darkly staining heme band which is not removed by antibody has catalase activity.

oxidase CRM from livers of tungsten-treated rats was attempted, using the purification scheme established for native enzyme (5). In initial attempts at purification, the heat step was omitted to allow for possible thermal instability of the sulfite oxidase CRM. However, it was subsequently established that the CRM is not destroyed by the heat step and that better purification is achieved if this step is included. The small amount of residual active enzyme in the livers was monitored during fractionation steps, and at each stage, the major portion of the CRM copurified with the active protein. Table II compares the purification of CRM from livers of tungsten-treated animals with the purification of native enzyme from control rat liver. As can be seen, the specific activity of the CRM preparation is consistently less than 3% of that of control enzyme at the corresponding stage of purity. As shown in Fig. 6, the material which is purified from livers of tungsten-treated rats is indeed the immunologically identical CRM as originally identified in these livers.

Properties of Sulfite Oxidase CRM: Prosthetic Groups—Table III compares the cofactor content of purified CRM with that of native enzyme. Sulfite oxidase CRM was found to contain the full complement of heme iron; therefore, the molybdenum and tungsten contents are presented as molybdenum to heme and tungsten to heme molar ratios. As can be seen, the molybdenum content of purified CRM is extremely low. In fact, molybdenum is detected to an extent directly reflecting the presence of residual active enzyme in the preparation. The observations that sulfite oxidase CRM copurifies with active enzyme and that it contains heme but not molybdenum suggest that the CRM is in fact molybdenum-free sulfite oxidase, inactive because it lacks this essential prosthetic group. Further studies on the purified CRM, reported below, establish the validity of this conclusion. As shown by the data in Table III, the presence of tungsten in the molybdenum-free enzyme preparation has been clearly established. As many as 35% of the enzyme molecules are seen to contain tungsten. Furthermore, a tight association of the metal with the protein is evident from the fact that the tungsten remains with the enzyme throughout the purification procedure. Fig. 7 documents the reliability of the technique used to quantitate the tungsten content of purified CRM. As can be seen, the tungsten content of the sample was well above the detection limit of the method and within a range of concentrations yielding a linear standard curve. Moreover, the absorption spectrum obtained from the sample is identical with that obtained from a dithiol complex of a tungsten standard. This demonstrates that the molybdenum content of the sample is too low to perturb the tungsten-dithiol spectrum to any degree and consequently does not affect the tungsten quantitation.

Data are also presented in Table III on the cofactor content of immune precipitates of native and molybdenum-free sulfite oxidase. These were prepared from high speed supernatants of hypotonic liver homogenates as already described. The values obtained for the immune precipitates are in good agreement with those observed with purified preparations and rule out the possibility that loosely associated metals in either the active enzyme or the CRM were lost during the purification procedure.

The presence of tungsten in a relatively large proportion of the molybdenum-free sulfite oxidase molecules raised the interesting question of whether those molecules which contain tungsten possess a small amount of sulfite oxidase activity or whether they are totally inactive. In the latter case, the approximately

![Table I: Quantitation of sulfite oxidase CRM in livers of tungsten-treated rats](http://www.jbc.org/)

| Experiment | Animal       | Sulfite oxidase iron or CRM a | Sulfite oxidase iron or CRM b |
|------------|--------------|-------------------------------|-------------------------------|
| 1          | Control      | 0.114                         | 0.118                         |
| 2          | Control      | 0.118                         | 0.122                         |
| 3          | Tungsten-treated | 0.078           | 0.080                         |
| 4          | Tungsten-treated | 0.079           | 0.082                         |

* Based on a millimolar extinction of 21.33 for ΔA (179.14) of the reduced minus oxidized difference spectrum of the pyridine heme chromogen of sulfite oxidase. 

* Assuming 0.900 μg of iron per mg of protein.
TABLE II

**Purification of sulfite oxidase and CRM from rat liver**

| Step | Control sulfite oxidase | Sulfite oxidase CRM | Specific activity of CRM/specific activity of control |
|------|-------------------------|---------------------|-----------------------------------------------------|
|      | Activity | Protein | units/mg | Activity | Protein | units/mg | %     |
| 1. Homogenate | 19,575 | 21,816 | 0.90 | 350 | 28,000 | 0.0125 | 1.4 |
| 2. Heat step | 18,055 | 7,636 | 2.36 | 200 | 8,932 | 0.0325 | 1.4 |
| 3. Ammonium sulfate fractionation | 20,100 | 5,040 | 3.99 | 312 | 5,600 | 0.0569 | 1.4 |
| 4. Acetone fractionation | 13,800 | 672 | 20.4 | 240 | 698 | 0.349 | 1.7 |
| 5. DEAE-cellulose chromatography | 0.630 | 9.49 | 699 | 86 | 7.28 | 11.8 | 1.7 |
| 6. Sephadex G-200 chromatography | 600 | 0.608 | 987 | 12 | 0.448 | 20.8 | 2.7 |

* Estimated value. Assays were performed under optimal conditions; however, the very low concentration of active enzyme in these preparations precluded accurate determinations.

![Fig. 6](image)

**Fig. 6.** Ouchterlony double immunodiffusion of purified rat liver sulfite oxidase and sulfite oxidase CRM. Purified proteins at a concentration of about 0.5 mg per ml were used. Wells 1, 2, and 4, native enzyme; Wells 3 and 5, sulfite oxidase CRM; center well, antibody prepared against purified control rat liver sulfite oxidase. Plates were washed, dried, and stained as in Fig. 2.

**TABLE III**

**Cofactor content of native sulfite oxidase and sulfite oxidase CRM**

Results are based on a millimolar extinction of 21.33 for $\Delta A_{457-545}$ of the reduced minus oxidized difference spectrum of the pyridine hemochromogen of sulfite oxidase.

| Purified preparations | Immune precipitates |
|-----------------------|---------------------|
| Molybdenum: home     | Tungsten: home      |
| Control               | 0.828               | 0.347               |
| CRM                   | 0.019               | 0.352               |

* No tungsten detected.

**TABLE IV**

**Quantitation of sulfite-reducible molybdenum in purified native and CRM preparations of sulfite oxidase**

| Purified preparations | Activity | Units/ml | Amplitude of g = 1.97 EPR signal |
|-----------------------|----------|----------|---------------------------------|
| Native sulfite oxidase | 0.101 | 130 | 14.8 |
| Sulfite oxidase CRM   | 5.75    | 115     | 15.6 |
| Ratio (CRM to native) | 57      | 0.88    | 1.05 |

were diluted to contain equal cytochrome c reductase activity. Since the CRM was about 2% active, this meant that the CRM, matched in activity to the control, was approximately 50 times more concentrated in terms of sulfite oxidase protein. The two preparations were then examined by EPR spectroscopy to quantitate the amount of sulfite-reducible molybdenum present in each. As can be seen, sulfite-reducible molybdenum in the CRM preparation fully accounted for its activity. Thus, it is possible to conclude that molybdenum-free sulfite oxidase is inactive, whether it exists as the apomolecule or has tungsten incorporated.

The tungsten center in the enzyme has been visualized by EPR spectroscopy as seen in Fig. 8. Addition of dithionite to a preparation of purified sulfite oxidase CRM produced a signal at $g = 1.87$ which we attribute to tungsten (V). The line shape of
the signal obtained bears a strong resemblance to that of molybdenum (V) in native sulfite oxidase in phosphate buffer (5). However, the EPR susceptibility of the tungsten center differed from that of the molybdenum center in the following respects: (a) the intensity of the tungsten (V) signal was unaltered on prolonged exposure to high concentrations of dithionite which abolish the molybdenum (V) signal (14), and (b) the power saturation behavior of the tungsten signal was quite different from that of the molybdenum signal.

Absorption Spectra—Fig. 9 presents the absorption spectra of purified native sulfite oxidase and molybdenum-free enzyme in the absence of any reductants. The spectra of the oxidized preparations are strikingly similar; only a careful examination of the different spectrum reveals features of dissimilarity (Fig. 9). The most obvious feature of the difference spectrum is a perturbation of the heme chromophore. The molybdenum free preparation shows increased absorbance in the Soret region which may reflect a more relaxed conformation of the protein near this prosthetic group. The difference spectrum in the ultraviolet region also reveals conformational differences between the native and molybdenum-free proteins. Perturbations of hydrophobic aromatic residues are seen; in particular, the peak at 295 nm suggests alterations in the environment of tryptophan residues (15). The large positive peak in the difference spectrum at 250 nm reflects the decreased absorbance of the molybdenum-free protein in this region. Kagi and Vallee (16) have noted a strong increase in absorbance at 250 nm when cadmium is bound to metallothionein reflecting the formation of a large number of mercaptide bonds. Increased 250-nm absorbance in native sulfite oxidase relative to the molybdenum-free protein may similarly reflect a metal-sulfhydryl linkage which is not present in the apoprotein.

Fig. 10 shows the absorption spectra of both preparations in the presence of sulfite and dithionite as reducing agents. The native enzyme is fully reduced by sulfite in less than 1 min. In contrast, the molybdenum-free preparation is only very slowly reduced by sulfite. After 30 min only 30 to 40% of the molecules show the spectrum of a reduced bs heme. Addition of dithionite, however, fully reduces the sample yielding a spectrum identical to that of native enzyme reduced by sulfite or dithionite.

Polyacrylamide Gel Electrophoresis—Fig. 11 shows polyacryl-
Fig. 10. Absorption spectra of control (A) and molybdenum-free (B) sulfite oxidase in the presence of various reductants. ---, no reductant; -- - -, reduced with 1 mM sodium sulfite for 1 min (control) or 30 min (CRM); ...., dithionite reduced. Protein concentrations were about 170 μg per ml.

amide gels of purified native and molybdenum-free sulfite oxidase stained for protein, heme, and activity. The native enzyme shows a single band in each case. The molybdenum-free enzyme when stained for protein or heme shows a single intense band with a diffuse band extending below. The nature or origin of this diffuse band is not clear; however, a similar dark and diffuse pattern is obtained for the sulfite oxidase CRM when the proteins of the IM space are subjected to electrophoresis on acrylamide gels and stained for heme. Thus, it can be said that the diffuse band is not an artifact of the purification procedure. The small amount of active enzyme in the CRM preparation does produce an activity band which, as expected, corresponds in position to that of native enzyme. The migration of the major portion of the CRM to a position identical to that of native enzyme suggests the lack of gross alteration in the distribution of surface charges and is consistent with the identical ionophoresis of the two forms on DEAE-cellulose.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Fig. 12) shows a major band at a position corresponding to a subunit molecular weight of about 58,000 for both the native and the molybdenum-free preparations. In addition, antibody complexes of the two proteins derived from crude liver extracts and subjected to electrophoresis in the presence of sodium dodecyl sulfate show subunits with molecular weights of 58,000 in each case.

Antigenic Properties of Native Sulfite Oxidase—As described above, the antibody prepared against purified native sulfite oxidase was of extreme value in identifying, quantitating, and characterizing the apoenzyme found in tissues of tungsten-treated rats. In addition to this, however, the antibody has served as a tool in probing other aspects of sulfite oxidase. Experiments were performed to determine the extent of inhibition by the antibody of the various activities of sulfite oxidase. It has been determined that the antibody is sufficient to precipitate all of the sulfite oxidase from a solution, results in complete inhibition of sulfite → cytochrome c activity in the complex. As shown in Fig. 13, sulfite → O₂ activity is also inhibited by these concentrations of antibody. Sulfit e → ferricyanide activity, on the other hand, is not blocked by the presence of the antibody. The important implication of these data is that the site where electrons from sulfite enter the enzyme, which is believed to be the molybdenum (14), is free of antibody interference to interaction of the enzyme with sulfite. This conclusion is further supported by EPR spectroscopy done on the enzyme-antibody complex. As seen in Fig. 14 the molybdenum center of the enzyme in the complex is reduced by sulfite, and moreover, retains its sensitivity to the effects of pH and anion composition of the medium (5).

Finally, sulfite oxidase activity is known to be present in rat tissues other than the liver; these include kidney, small intestine, heart, and lung. While the enzyme has not been purified and characterized from these tissues, studies using the antibody prepared against hepatic sulfite oxidase have suggested that the enzyme in all rat tissues is identical. Extracts of liver, kidney, intestines, and heart were diluted to contain approximately 3.2 units of sulfite oxidase activity per ml. Incubation of each extract with sufficient antiserum to inhibit 66% of the liver enzyme was seen to inhibit the kidney, intestine, and heart enzymes to the same extent (Table V). In addition the number of units of lung sulfite oxidase inhibited per microliter of antiserum was identical with that seen in the other tissues. Thus, the population of inhibiting antibodies in the antiserum prepared against hepatic sulfite oxidase has equal affinity for the enzyme from other rat tissues, indicating a very strong degree of similarity between the sulfite oxidase proteins in the various tissues.

DISCUSSION

The production of deficiencies of sulfite oxidase and xanthine oxidase in the rat by the use of tungsten as an antagonist of molybdenum utilization has been reported in an earlier publication (1). In such a situation where the activity of an enzyme is lowered, the question arises as to whether this is a reflection of an actual shutdown of enzyme synthesis or, alternatively, whether the animal is continuing to synthesize the protein but in an inactive form. The experiments reported in this paper have shown that inactive sulfite oxidase is being made in considerable amounts by tungsten-treated rats. The protein synthesized is inactive because it lacks the essential molybdenum.
prosthetic group. Approximately 35% of the molecules have tungsten incorporated into the vacant molybdenum site. Tungsten-containing sulfite oxidase has no detectable cytochrome c reductase activity.

The inactive, molybdenum-free protein has been compared with the native sulfite oxidase in terms of subcellular localization, steady state content in liver, and thermal stability. By each of these criteria, the inactive enzyme is identical to the native. The molybdenum-free protein is packaged correctly in the IM space, is present in amounts not vastly different from native suggesting a similar pattern of turnover, and is stable to heating to 58° in the presence of sulfate. Other similarities between the two proteins include their apparent immunological identity, indistinguishable subunit molecular weights as determined by acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, and similar surface charge distributions as shown by co-electrophoresis on acrylamide gels and co-ionophoresis on DEAE-cellulose. The proteins also have similar absorption spectra, both in the oxidized and reduced forms, although the conditions required for reduction differ. The difference spectrum of the native enzyme minus the inactive protein revealed perturbations of the heme prosthetic group, and of hydrophobic aromatic residues. No absorbance change was detected in the visible region which could be attributed specifically to the molybdenum of the native enzyme. Exposure of a sulfhydryl residue was inferred in the inactive protein, however, from the difference peak at 250 nm, and the possibility has been raised that this reflects a perturbation of the molybdenum center.

The incorporation of tungsten into molybdenum-free sulfite oxidase is of particular interest. The presence of tungsten in nitrate reductase (17, 18) and nitrogenase (19) of plants and bacteria grown in the presence of high concentrations of the metal has been reported. Preliminary studies on the inactive sulfite oxidase, however, indicated that no tungsten was incorporated into the protein. The experimental conditions in the case where no tungsten was present in the protein differed in several respects from those described in this article. In the earlier studies animals were given a normal protein diet which was low in molybdenum (supplied by Nutritional Biochemicals). Tungsten was administered at 100 ppm in the drinking water, and 10 g of sucrose were added per liter to hide the taste of the tungsten salt. On this regimen, as mentioned, no tungsten was incorporated into sulfite oxidase, although the metal was transported to the liver (1). It would appear that the systems for the absorption and transport of molybdenum, presumably as molybdate, are sufficiently nonspecific to accept tungsten (as tungstate) in place of molybdenum. This is not surprising since the two metals not only have identical outer shell electronic configurations, but are also extremely similar in their atomic and ionic radii (20). Discrimination between the two metals, however, is effected at that stage where the absorbed molybdenum is activated or modified for incorporation into the protein.

Under the experimental conditions used for studies reported in this article, tungsten is again seen to be absorbed and transported to the liver, but to a considerably greater extent. Hepatic tungsten levels as high as 1.72 μg per g have been noted. Perhaps the observed incorporation of tungsten into sulfite oxidase under these conditions is a reflection of the higher tissue levels of the metal sufficient to overcome an unfavorable equilibrium for tungsten activation. It is not possible at the present time to say whether the increased tungsten accumulation and incorporation into sulfite oxidase is a reflection of the higher dose of tungsten administered, the mode of administration of the metal or other unknown factors resulting from minor differences in the compositions of the diets from the two suppliers. However, once the basis for the observed differences in hepatic and sulfite oxidase tungsten levels is defined, it may be possible to selectively produce molybdenum-free (apo) sulfite oxidase or
sulfite oxidase with tungsten specifically incorporated into the molybdenum site. It is expected that such preparations will be of extreme value in further investigations into the nature of the molybdenum center of rat liver sulfite oxidase.

The details of a simple and reliable colorimetric assay for tungsten and molybdenum have been outlined under "Experimental Procedure." The importance of such a method, especially for tungsten which is not amenable to analysis by atomic absorption spectroscopy, and its advantage over using radioisotopes in the type of work described are obvious.

The differential reduction of the heme in the native and molybdenum-free proteins by sulfite, as revealed in their absorption spectra, can be examined with regard to the presence of tungsten-containing molecules in the inactive preparation. The heme of native enzyme was seen to be fully reduced 1 min after the addition of sulfite; in fact, reduction may actually have been complete within a few seconds or less. In the inactive preparation a very slow rate of reduction was noted until a maximum of 30 to 40% of the heme had been reduced (about 30 min after the addition of sulfite). Since electrons from sulfite are believed to enter sulfite oxidase via the molybdenum center, with subsequent transfer to the heme, it seems likely that the extent of reduction of the heme of the inactive preparation reflects a very slow rate of flow of electrons through the tungsten center of those molecules which contain tungsten. The molybdenum-free (apo) molecules, on the other hand, cannot accept electrons from sulfite and thus remain fully oxidized. Clearly, the rate of electron flow through the tungsten center is of such a low magnitude that enzyme-catalyzed reduction of cytochrome c is not detectable in the conventional spectrophotometric activity assay. Earlier preparations of inactive sulfite oxidase, which contained no tungsten enzyme, were seen to be reduced by sulfite to an extent of less than 5%, fully accounted for by residual active enzyme in the preparation.

While the EPR properties of tungsten analogs of molybdenum proteins have not been described before, inorganic tungsten complexes have been studied extensively. As a rule tungsten (V) complexes have lower g values than corresponding molybdenum (V) derivatives (21). The observation that the g values of tungsten (V) and molybdenum (V) in sulfite oxidase are 1.87 and 1.97, respectively, is consistent with the findings with the inorganic complexes. The similarity of the line shapes of the EPR signals of the two metals in sulfite oxidase is evidence that they occupy the same site and suggests that the tungsten center of a suitable electron donor. Apparently the lower electronegativity of tungsten relative to molybdenum precludes efficient transfer of electrons from sulfite.

Some interesting aspects of sulfite oxidase have been revealed by antibody studies and are worthy of mention. First of all, a low molecular weight protein, containing b, heme, has been observed by Fukushima et al. (22) in the IM space of rat liver and described by them as an apparent proteolytic degradation prod-

![FIG. 13 (left). Inhibition of sulfite oxidase activities by anti-serum. O, sulfite → cytochrome c; □, sulfite → ferricyanide; D, sulfite → O₂.

![FIG. 14 (right). EPR spectra of sulfite oxidase-antibody complex. Aliquots of about 200 units of sulfite oxidase were precipitated from a high speed liver supernatant with antibody. The immune precipitates were washed, suspended in 0.5 ml of various buffers, and transferred to quartz EPR tubes. A, enzyme in 0.1 M Tris-HCl, pH 7.0, prior to addition of sulfite; B, enzyme in 0.1 M Tris-HCl, pH 7.0, reduced with 2.0 mM sodium sulfite for 1 min; C, enzyme in 0.1 M Tris-HCl, pH 9.0, sulfite-reduced; D, enzyme in 0.05 M potassium phosphate, pH 7.0, sulfite-reduced; E, sulfite oxidase CRM precipitated from an equal volume of high speed supernatant from liver of a tungsten-treated rat, in 0.1 M Tris-HCl, pH 7.0, sulfite-reduced. EPR conditions were modulation amplitude, 10 G; microwave frequency, 9.12 GHz; microwave power, 5 milliwatts; time constant, 1 s; receiver gain, 10,000; and scan rate, 65.2 G per min.

| Tissue                  | Homogenate ratio | Activity            | Inhibition | Units inhibited per ml of serum |
|-------------------------|------------------|---------------------|------------|-------------------------------|
|                         |                  | Before incubation   | After incubation | %                       |
| Liver                   | 1:30             | 3.25                | 1.12       | 66                            | 0.26                 |
| Kidney                  | 1:12             | 3.17                | 1.87       | 63                            | 0.24                 |
| Small intestine         | 1:4              | 3.42                | 1.28       | 63                            | 0.26                 |
| Heart                   | 1:1              | 3.22                | 1.08       | 66                            | 0.26                 |
| Lung                    | 1:1              | 0.95                | 0.52       |                               | 0.22                 |
uct of sulfite oxidase. The presence of this small $b_5$ protein has been confirmed in our studies as can be seen in Fig. 5. IM preparations from both control and tungsten-treated rats contain a heme protein which is seen very near the dye front on acrylamide gels. After incubation of the IM space preparations with antibody prepared against sulfite oxidase, and centrifugation to remove any precipitated material, this small heme protein is no longer seen near the dye front, thus indicating its cross-reactivity with sulfite oxidase. Interestingly, however, a new heme band appears near the top of the gel after such treatment (not visible in Fig. 5). If the small $b_5$ portion of sulfite oxidase has only one of the antigenic determinants, this new band may correspond to an antibody molecule complexed to 2 molecules of the small $b_5$ protein. Such an antibody complex would not have been removed by centrifugation at 35,000 $\times g$ yet would be of sufficient size to have lower mobility in 7.5% polyacrylamide gel.

A differential inhibition of the various activities of native sulfite oxidase in the presence of the antibody has been described. The interaction of cytochrome $c$ with the enzyme is apparently obstructed in the antigen-antibody complex, while the smaller ferricyanide molecule has free access and can accept electrons with no interference. Reduction of oxygen, on the other hand, is impaired in the presence of antibody, suggesting perhaps that egress of electrons to this acceptor occurs from a site different from that of ferricyanide interaction. The availability of the sulfite binding site even in the presence of antibody has been established by several criteria. Uninhibited sulfite to ferricyanide activity, rapid reducibility of the molybdenum center by sulfite as seen by EPR, and anion sensitivity of the molybdenum center in the enzyme-antibody complex all suggest that sulfite has free access to the molybdenum center of the enzyme in the complex.

A technique for producing molybdenum-free sulfite oxidase in vitro has recently been developed in this laboratory (23). Treatment of purified chicken liver sulfite oxidase with arsenite quantitatively removes the molybdenum from the protein. It will be of considerable interest to extend this work to the rat enzyme and to do comparative studies on the molybdenum-free proteins prepared by the two very different approaches.

Acknowledgments—The authors thank Dr. Harvey Sage for instruction in basic immunological techniques and assistance in early phases of the antibody studies. H.J.C. is grateful to Dr. J. R. Fouts for his interest, encouragement, and support.

REFERENCES
1. Johnson, J. L., Rajagopalan, K. V., and Cohen, H. J. (1974) J. Biol. Chem. 249, 859-866
2. Johnson, J. L., Waud, W. R., Cohen, H. J., and Rajagopalan, K. V. (1974) J. Biol. Chem. 249, 5056-5061
3. Cohen, H. J., and Fridovich, I. (1971) J. Biol. Chem. 245, 359-366
4. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, H. J. (1951) J. Biol. Chem. 203, 265-275
5. Kessler, D. L., Johnson, J. L., Cohen, H. J., and Rajagopalan, K. V. (1974) Biochim. Biophys. Acta 324, 86-96
6. Bickford, C. F., Jones, W. S., and Keene, J. S. (1948) J. Amer. Pharm. Ass. 37, 255-261
7. Clark, I. J., and Axley, J. H. (1955) Anal. Chem. 27, 2000-2003
8. Falk, J. E. (1964) Porphyrins and Metalloporphyrins, p. 181, Elsevier Publishing Co., Amsterdam
9. Davis, B. J. (1964) Ann. N. Y. Acad. Sci. 121, 404-427
10. Hau, A., Tudhope, G. R., Cartwright, G. E., and Winning, M. M. (1962) J. Clin. Invest. 41, 570-587
11. Cohen, H. J. (1973) Anal. Biochem. 53, 208-222
12. Weber, K., and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412
13. Cohen, H. J., Butcher-Lange, S., Kessler, D. L., and Rajagopalan, K. V. (1972) J. Biol. Chem. 247, 7759-7766
14. Cohen, H. J., Fridovich, I., and Rajagopalan, K. V. (1971) J. Biol. Chem. 246, 374-382
15. Donovan, J. W., Laskowski, M., Jr., and Scheraga, H. A. (1958) Biochim. Biophys. Acta 29, 455-456
16. Kagi, J. H. R., and Walbee, B. L. (1961) J. Biol. Chem. 236, 2435-2442
17. Nottton, B. A., and Hewitt, E. J. (1971) Biochem. Biophys. Res. Commun. 44, 702-710
18. Eakers, A., Ysha, J. M., Cardenas, J., Herrera, J., Aparacio, P. J., and Losada, M. (1972) Plant Cell Physiol. 13, 175-178
19. Benemann, J. R., Smith, G. M., Kostel, P. J., and McKenna, C. E. (1972) Fed. Eur. Biochem. Soc. Lett. 29, 219-221
20. Heslop, R. B., and Robinson, P. L. (1960) Inorg. Chem. 71, 417-431
21. Kon, H., and Sharpless, N. F. (1966) J. Phys. Chem. 70, 105-109
22. Fukushima, K., Ito, A., Omura, T., and Sato, R. (1972) J. Biochem. 71, 417-461
23. Kessler, D. L. (1973) Ph.D. thesis, Duke University
Molecular Basis of the Biological Function of Molybdenum: MOLYBDENUM-FREE SULFITE OXIDASE FROM LIVERS OF TUNGSTEN-TREATED RATS

Jean L. Johnson, Harvey J. Cohen and K. V. Rajagopalan

J. Biol. Chem. 1974, 249:5046-5055.

Access the most updated version of this article at http://www.jbc.org/content/249/16/5046

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/249/16/5046.full.html#ref-list-1