Hepatic Sulfite Oxidase

A FUNCTIONAL ROLE FOR MOLYBDENUM*

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SUMMARY

Sulfite oxidase (EC 1.8.3.1), when reduced by sulfite, exhibited the electron paramagnetic resonance spectrum of molybdenum(V). Specific colorimetric analyses demonstrated that the purified enzyme contained 1 molybdenum per heme.

The electron paramagnetic resonance signal of molybdenum, which was generated by the addition of sulfite, was enriched by the purification procedure to the same degree as was the sulfite-reducible heme and the sulfite oxidase activity. Incubation of the enzyme with \( 5 \times 10^{-3} \text{M} \) arsenite produced parallel losses of the sulfite-inducible molybdenum signal and the catalytic activity.

Incubation of the enzyme with cyanide in the presence of sulfite resulted in slow, progressive, and irreversible inactivation. This treatment also caused a concomitant and proportional loss of the molybdenum signal. Cyanide had no effect on the absorption spectrum of the enzyme in the visible or near ultraviolet regions of the spectrum.

Whereas the heme prosthetic group of the enzyme was oxidized in the steady state achieved by the simultaneous presence of sulfite and ferricyanide, the molybdenum component was in the reduced (pentavalent) state.

Examination of the molybdenum signal as a function of pH, showed a shift in the field strength of maximum energy absorption, and a change in the shape of the signal. The electron paramagnetic resonance spectra of the enzyme in D_2O at pH 7.5 and at pH 9.6 demonstrated that the environment of the unpaired electron was axially symmetrical at the lower pH but was asymmetrical at the more alkaline pH.

indications of another electron transport component on the enzyme which was the site of reduction of 1-electron acceptors. Thus, anions inhibited the reduction of ferricyanide and cytochrome c, but not of oxygen. Inhibition of one step in a process can inhibit the over-all process only when the affected step is rate limiting. Hence, the transfer of electrons from some component of the enzyme to ferricyanide must have been slower than the transfer of electrons from sulfite to that component. It follows that the group mediating transfer of electrons from sulfite to ferricyanide must be reduced in the steady state. However, the heme prosthetic group of sulfite oxidase was found to be oxidized in the combined presence of sulfite and of ferricyanide (2). Hence, some prosthetic group other than heme must have been functioning in electron transfer. The visible and ultraviolet spectra of the enzyme gave no indications for the existence of a chromophoric component other than heme. However, low temperature electron paramagnetic resonance spectroscopy revealed the presence of molybdenum(V) in sulfite oxidase reduced with sulfite.

There are only a few enzymes which have been shown to contain molybdenum. In mammalian tissues only xanthine oxidase (3) and aldehyde oxidase (4) have thus far been shown to contain functional molybdenum. These enzymes have similar prosthetic groups, molecular weights, and substrate specificities (3, 4). There are two additional, nonmammalian, molybdenum-containing enzymes. These are the nitrate reductase of bacteria and plants (9), and the molybdoferredoxin of the nitrogenase systems of Azotobacter vinelandii (6) and Clostridium pasteurianum (7). All of these molybdo enzymes contain additional prosthetic groups. Thus xanthine oxidase and aldehyde oxidase contain FAD and nonheme iron (3, 4), nitrate reductase contains FAD (8) and heme (9), and molybdoferredoxin contains nonheme iron (10). In only one of these enzymes was it reportedly possible to reversibly remove the molybdenum with parallel loss and restoration of enzymic function (11).

Sulfite oxidase is thus the fifth enzyme shown to contain molybdenum, and only the third mammalian enzyme in this category. Sulfite oxidase exhibits the absorption spectrum of a cytochrome b, and its substrate specificity excludes the substrates of xanthine oxidase, aldehyde oxidase, nitrate reductase, and nitrogenase (1). This paper documents the presence and function of molybdenum in sulfite oxidase, and describes some aspects of the behavior of its EPR signal which should utiliz...
mately be useful in elucidating the environment of this molybdenum.

MATERIALS AND METHODS

Deuterium oxide (99.8%), was purchased from Volk Radiochemical Company, New York. D(-)-Phenylglycine was obtained from Aldrich and was recrystallized twice from boiling water. D-Amino acid oxidase was obtained from Worthington as the hog kidney acetone powder. Xanthine oxidase was prepared from cow's milk by the method of Brady (12). All other materials and methods were as described in the preceding papers (1, 2).

EPR spectroscopy was performed with a Varian model E-9 HF EPR spectrometer, with a 9.5 GHz microwave bridge assembly, and operated at a modulation frequency of 100 KHz. Parameters such as the time constant, gain, modulation amplitude, temperature, microwave power, and microwave frequency were varied and are specified in each case. Samples of sulfate oxidase were prepared for EPR spectroscopic analysis by mixing 0.2 ml of the enzyme and 0.02 ml of 0.10 M sodium sulfate in a 3 mm inner diameter, 12 cm long Quartz tube for 1 min, and then freezing the samples in liquid nitrogen. Samples of xanthine oxidase were similarly prepared by mixing 0.1 ml of the enzyme with 0.1 ml of 1.5 x 10^-3 M xanthine. The EPR signals were integrated by the summation method of Ayscough (13). The areas under the integrated EPR absorption curves were arrived at by drawing the curves on uniform density graph paper, and then cutting out and weighing the spectra so obtained.

Molybdenum was quantitated by a minor modification of the method of Clark and Asley (14). Enzyme samples (2 to 4 mg) were processed along with standards containing 0.25 to 5.0 µg of molybdenum. The green color yield, read at 680 nm, was proportional to the amount of molybdenum in this range. Agreement between duplicates was excellent.

Assays for total flavin were performed by the fluorimetric method of Burch, Bessey, and Lowry (15), while FAD was specifically assayed for, in boiled extracts of sulfite oxidase, by an enzymatic method involving the measurement at 592 nm of the oxidation of d(-)-phenylglycine (16) by apo-d-amino acid oxidase (17).

The pH was measured at 25°C with a Radiometer model 26 pH meter. Values of pH were arrived at by adding 0.4 pH unit to the readings obtained with the glass electrode (18).

RESULTS

EPR Evidence for Molybdenum in Sulfite Oxidase—Sulfite oxidase, which was 4.8 x 10^-4 M with respect to its heme prosthetic group, was dissolved in 0.10 M Tris-HCl, 1 x 10^-4 M EDTA, pH 7.0, and was frozen at the temperature of liquid nitrogen. When scanned from 0 to 10,000 gauss, at a microwave power up to 100 mwatts, receiver gain of 50,000, this enzyme gave no indication of the presence of paramagnetic centers. However, when the enzyme was treated with 0.01 M sulfite for 1 min prior to freezing, it exhibited a strong asymmetric EPR signal at g = 1.970. This EPR signal, illustrated in Fig. 1, is similar to those obtained from xanthine oxidase (10) and aldehyde oxidase (20) in the presence of their respective substrates, and is attributed to pentavalent molybdenum. The height of the signal shown in Fig. 1 was found to be proportional to the concentration of sulfite oxidase and, as expected for molybdenum, it was abolished by reduction with excess dithionite (20).

![EPR spectrum of reduced sulfite oxidase. Sulfite oxidase (48 µg with respect to heme), in 0.10 M Tris-HCl, pH 7.0, was mixed with 0.01 M sulfite and frozen in liquid nitrogen as described under "Materials and Methods." The conditions under which this spectrum was obtained were, temperature = -100°C, time constant = 0.3 sec, modulation amplitude = 2 gauss, gain = 2500, power = 5 mwatts, and frequency = 9.0815 GHz. The g, calculated from this spectrum was 1.970.](http://www.jbc.org/)

**Table I**

<table>
<thead>
<tr>
<th>Cofactor</th>
<th>Enzyme</th>
<th>µmoles/mg</th>
<th>µmoles/mole</th>
</tr>
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<tbody>
<tr>
<td>Molybdenum</td>
<td>17.7</td>
<td>2.06</td>
<td></td>
</tr>
<tr>
<td>Heme</td>
<td>16.7</td>
<td>1.92</td>
<td></td>
</tr>
<tr>
<td>FAD</td>
<td>&lt;0.8</td>
<td>&lt;0.09</td>
<td></td>
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</tbody>
</table>

EPR signal elicited by adding sulfite to sulfite oxidase was the result of a very specific interaction between this substrate and this enzyme, since sulfite did not bring forth a comparable signal from xanthine oxidase and xanthine did not generate such a signal when added to sulfite oxidase.

**Colorimetric Quantitation of Molybdenum—**Chemical analyses for molybdenum were performed on the purified sulfite oxidase. The heme component was also quantitated in terms of its pyridine-hemochromogen (2), and the ratio of molybdenum to heme was found to be 1.05. These results are given in Table I. Fluorimetric analyses (15) for flavins and specific enzyme analyses for FAD (16, 17) indicated that the purified enzyme did not contain significant amounts of flavin. Furthermore, neither FAD nor FMN, alone or in combination, had any effect on the activities of sulfite oxidase.

**Copurification of Molybdenum and Enzymatic Activity—**Since the EPR signal of molybdenum was specifically elicited by sulfite, it seemed likely that molybdenum was a prosthetic group of sulfite oxidase. In order to obtain additional evidence for this supposition, the enrichment with respect to both EPR signal and enzymatic activity was measured as a function of the degree of purification of the enzyme. The specific molybdenum signal was taken to be the height of the signal per mg of protein. At least 800 units of enzyme per ml were required for the molybdenum signal to be detectable. This necessitated concentration...
of the cruder fractions by ultrafiltration in collodion membranes prior to measurement of the sulfite-elicited molybdenum signal. As demonstrated in Fig. 2, the specific molybdenum signal and the specific enzymatic activity increased in parallel over a 340-fold range of purification. Furthermore, it was shown that during the latter stages of purification, over a 25-fold range, the amount of sulfite-reducible heme per mg of protein, as measured at 423 nm, also paralleled the increase in specific molybdenum signal.

**Effect of Arsenite on Enzymatic Activity and on Molybdenum Signal**—Arsenite inhibits xanthine oxidase and aldehyde oxidase (21) and is thought to do so by acting on the molybdenum prosthetic groups of these enzymes (22). The effect of arsenite on sulfite oxidase was investigated in order to further explore the relationship between the molybdenum and the enzymatic activity. When sulfite oxidase was incubated with $5 \times 10^{-2}$ M arsenite, the activity of the enzyme toward both oxygen and cytochrome c declined. This inactivation was first order with respect to residual activity, and the pseudo first order rate constant was 0.013 min$^{-1}$ when oxygen was the electron acceptor, and 0.015 min$^{-1}$ when cytochrome c was the electron acceptor. The effect of arsenite on the amplitude of the sulfite-elicited EPR signal of the enzyme was also measured and, as shown in Fig. 3, the latter declined in parallel with the catalytic activities. The shape of the molybdenum signal was not altered by arsenite.

**Effect of Cyanide on Sulfite Oxidase**—Cyanide (0.01 M) did not inhibit sulfite oxidase when added to reaction mixtures at their inception or when incubated with the enzyme for several hours prior to initiation of the assay reactions (2). However, incubation of the enzyme with cyanide in the presence of sulfite did cause a gradual loss of catalytic functions. This is illustrated in Fig. 4. Oxygen had no effect on this sulfite-dependent inactivation by cyanide, and the visible spectrum of the sulfite-reduced enzyme was unaltered even after 90% of the activity had been lost. Dialysis of the enzyme in the presence or absence of sulfite failed to restore the lost activity. The effect of incubation of the enzyme with cyanide plus sulfite on the sulfite-elicited molybdenum signal was found to parallel its effect on catalytic activity. This is illustrated by the data in Fig. 5. When the data in Fig. 5 were plotted on semilogarithmic coordinates, a straight line was obtained from whose slope a pseudo first order rate constant of 0.0076 min$^{-1}$ was obtained.

**Effect of Methanol**—Because methanol has been shown to gradually inhibit both xanthine oxidase and aldehyde oxidase (21) when present in their respective reaction mixtures, its effect on sulfite oxidase was explored. However, 1.5 M methanol was without effect on the sulfite-cytochrome c reduction assay of sulfite oxidase, whether this assay was performed at pH 7.8 in a phosphate buffer or at pH 8.5 in a Tris-HCl buffer.

**Steady State of Molybdenum in Presence of Sulfite and Ferricyanide**—In the previous paper (2), it was demonstrated that the active fractions of sulfite oxidase were eluted in 0.01 M sodium carbonate, 0.01 M sodium cyanide, and 0.005 M sodium sulfite at pH 10.0 and 25° in the absence of oxygen. At intervals aliquots were assayed for sulfite → cytochrome c (O and •) and sulfite → oxygen (□ and □) reductase activities.

**Fig. 3 (left).** The effect of arsenite on enzymatic activity and on the Mo(V) EPR signal. Sulfite oxidase was incubated in 0.1 M Tris-HCl containing 0.006 M sodium arsenite at 25° and pH 8.5. At intervals aliquots were assayed for sulfite → cytochrome c (□—□) and sulfite → oxygen (□—□) reductase activities. At 0, 60, and 120 min, 0.2-ml samples were withdrawn and quantitated for the height of the sulfite-elicited EPR signal (X------X).

**Fig. 4 (center).** The effect of cyanide on sulfite oxidase activity. Enzyme was incubated in 0.1 M Tris-HCl containing 0.01 M sodium cyanide at pH 8.5 and 25° in the presence (open symbols) and in the absence (closed symbols) of 0.005 M sodium sulfite. At intervals aliquots were assayed for sulfite → cytochrome c (□ and □) and sulfite → oxygen (X and •) reductase activities.

**Fig. 5 (right).** Congruous effects of cyanide on activity and EPR signal. Enzyme was incubated in 0.10 M sodium carbonate, 0.01 M sodium cyanide, and 0.005 M sodium sulfite at pH 10.0 and 25° in the absence of oxygen. At intervals aliquots were assayed for sulfite → cytochrome c reductase activity (X) and for the height of the sulfite-elicited EPR signal (□). The latter was corrected for the changes in Mo(V) signal when enzyme was similarly treated in the absence of cyanide.
respects to that which gave Spectrum B'B except that it contained
2 mwatts, and frequency = 9.052 GHz. In that instant = 0.3 sec, modulation amplitude = 4 gauss, power = 2
heme of sulfite oxidase was fully oxidized in the steady state
achieved in the combined presence of sulfite and ferricyanide.
On the basis of the ability of anions to inhibit the reduction of
cyanide and cytochrome c without inhibiting the reduction of
ferricyanide, it was reasoned that the group on the enzyme, from
which electrons flowed to these l-electron acceptors, was reduced.
Could molybdenum qualify for this role? In order to do so, the
molybdenum of sulfite oxidase would have to be reduced in the steady
state reached in the combined presence of sulfite and of
ferricyanide. The necessity for high concentrations of enzyme
combined with the high rate of turnover in the sulfite → ferricyanide
reaction presented a technical impediment to the desired
EPR measurements. This was circumvented by using chloride
molybdenum of sulfite oxidase would have to be reduced in the
presence of sulfite and ferricyanide. Such effects have been ascribed to spin-lattice interactions (23).

When the microwave power was set at a level well below saturation even at the lowest temperatures studied, the signal height
was measured as a function of temperature. When log signal
height was plotted as a function of reciprocal absolute temperature, the data generated two line segments which intersected
at -113°. The molybdenum EPR signals of sulfite oxidase and of xanthine oxidase responded similarly indicating that in both enzymes the environment of the molybdenum undergoes a transition centered at approximately -113°. Fig. 8 illustrates these results. The EPR signal of a cupric-EDTA standard, when
investigated, gave a slope similar to that shown by the enzyme above 113° and did not exhibit a transition in this temperature
range. There was no significant broadening of the Mo(V) signal
at the higher temperatures.

Comparison of Intensities of Molybdenum Signals of Sulfite Oxidase and Xanthine Oxidase—It has been reported that EPR
spectroscopy detects only 37% of the molybdenum in xanthine
oxidase. This estimate was based on a quantitation by com-
parison to a copper-EDTA standard (24). The intensity of the
molybdenum(V) signal from sulfite oxidase was compared to that of xanthine oxidase. Thus each enzyme, reduced by its respec-

FIG. 6. The oxidation state of molybdenum in the combined
presence of sulfite and ferricyanide. In a quartz EPR tube, 0.02
ml of 1.0 M sodium sulfite was mixed with 0.2 ml of enzyme in 0.10 M
Tris-HCl, pH 9.0, and 2.0 M KCl, and after 1 min the mixture was
frozen in liquid nitrogen and the EPR signal was recorded (A).
The mixture was then thawed, 0.02 ml of 1.0 M potassium ferricyanide
was added, and after immediate refreezing, the EPR spectrum was again recorded (B'). The mixture was again
thawed, was maintained at 25° until all of the ferricyanide was
reduced, and after refreezing the EPR spectrum was recorded (C).
Spectrum D was recorded from a mixture which was similar in all
respects to that which gave Spectrum B'B except that it contained
no enzyme. EPR spectra were recorded at -100°, time constant
= 0.3 sec, modulation amplitude = 4 gauss, power = 2
mwatts, and frequency = 9.052 GHz. In A, B, and C gain was set
at 5000 while in B' and D it was set at 500.

FIG. 7. The effect of temperature on the saturation curve for
the EPR signal. The sulfite-elicited EPR spectrum of Mo(V) was
recorded at -191° (I), -100° (2), -75° (3), -50° (4), and at -25°
(5). Microwave power was varied from 0.5 mwatts to 200 mwatts.
Signal heights were normalized to the same gain. S represents
signal height and P represents microwave power. Deviation from
the horizontal indicates saturation.
EPR signal heights, normalized with respect to gain, were measured at 0.5 mwatts power as a function of temperature and the natural log of the signal height is plotted as a function of reciprocal absolute temperature. The upper curve (■—■) presents the data obtained with xanthine oxidase while the lower curve (○—○) gives that obtained with sulfite oxidase. The steep slopes, seen in the high range of temperature, were 206 and 250 for xanthine oxidase and sulfite oxidase, respectively, while the corresponding smaller slopes, seen at the lower temperatures, were 76 and 90. The intersection of the extrapolated steep and shallow slopes was at \(-113^\circ\). For comparison, the data obtained from a cupric-EDTA standard defined a line whose slope was 227 over this entire temperature span.

**Table II**

<table>
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<tr>
<th>pH</th>
<th>Field strength maximum (Gauss)</th>
<th>Values for Mo(V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.21</td>
<td>3281.5</td>
<td>1.971</td>
</tr>
<tr>
<td>7.58</td>
<td>3282.5</td>
<td>1.971</td>
</tr>
<tr>
<td>8.00</td>
<td>3285</td>
<td>1.971</td>
</tr>
<tr>
<td>8.40</td>
<td>a 3287, b 3285.5</td>
<td>1.963</td>
</tr>
<tr>
<td>8.82</td>
<td>3297</td>
<td>1.962</td>
</tr>
<tr>
<td>9.21</td>
<td>3297</td>
<td>1.962</td>
</tr>
</tbody>
</table>

* Data compiled from Fig. 9A.
Fig. 10. The effects of pH on the integrated Mo(V) EPR signal of sulfite oxidase. The EPR signals presented in Fig. 9A were integrated as described under “Materials and Methods,” and the resultant absorption curves plotted as a function of field strength. These curves represent EPR signals obtained at pH 7.21 (1), pH 7.58 (2), pH 8.00 (3), pH 8.40 (4), pH 8.82 (5), and pH 9.2 (6). The isosbestic point of these curves is at 3289.5 gauss and gave a value of \( g = 1.967 \).

Effects of D\(_2\)O on Acidic and Basic EPR Spectra of Sulfite Oxidase—As shown in Fig. 9, the EPR signal of sulfite oxidase exhibited two doublets at lower pH which were not evident at the higher pH. It appeared possible that these doublets were occasioned by the splitting effect of the dissociable proton, whose titration curve was shown in Fig. 9B. If this were the case, then the replacement of H\(_2\)O by D\(_2\)O at the acid extreme of pH would convert each doublet into a singlet; whereas D\(_2\)O would have no effect at the alkaline pH. That this was the case is shown by the data in Fig. 11. Fig. 11.4 presents the EPR spectra of reduced sulfite oxidase at pH 7.5 and at pH 7.5 in water and in D\(_2\)O, respectively. It is clear that replacement of H\(_2\)O by D\(_2\)O did eliminate the doublets. The spectrum seen in D\(_2\)O at pH 7.5 is that of an unpaired spin in an axially symmetrical configuration, where \( g_1 = 2.000 \) and \( g_\perp = 1.998 \). In contrast, at the alkaline pH, replacement of H\(_2\)O by D\(_2\)O was without effect on the EPR signal. The EPR spectrum of sulfite oxidase at pH 10.0 is that of an unpaired spin in an asymmetrical ligand field where \( g_1 = 1.984, g_\perp = 1.961, \) and \( g_\parallel = 1.950 \). It is clear that removal of the proton from the vicinity of the molybdenum by raising the pH changes the ligand field of the molybdenum(V) from one of axial symmetry to an asymmetrical one. The proton coupling constants derived from the EPR spectrum at pH 7.0 in 0.10 M Tris-HCl (Fig. 1) were \( a_{\perp} = 10 \) gauss and \( a_{\parallel} = 12 \) gauss.

Hyperfine Splitting Constants for Molybdenum Signal—Naturally occurring molybdenum contains 26\% of isotopes \(^{95}\)Mo and \(^{97}\)Mo, which have nuclear moments of 5/2, and which should split each part of the EPR signal of sulfite oxidase into six components. In order to simplify the EPR signal, the splitting due to the dissociable proton was eliminated by dialyzing the enzyme against 0.10 M Tris-DCI at pH 7.0. When this was not done,
the proton splitting was seen to be superimposed upon the splitting due to the nuclear moments of the molybdenum isotopes. The enzyme in D2O was reduced with sulfite, and its EPR spectrum was recorded. The gain was then increased 4-fold, and the modulation amplitude was increased 5-fold, and the wings of the EPR spectrum were again recorded to exaggerate the results of the hyperfine splitting. Fig. 12 illustrates the spectra so obtained. The hyperfine splitting constants were found to be $a_{\text{h}}^{\text{Mo}} = 62.5$ gauss and $a_{\text{h}}^{\text{Mo}} = 45.5$ gauss. The small signal just to the right of the main signal, seen more clearly in the amplified spectrum, was probably due to that small proportion of the enzyme at pH = 9.6 was more complex than that shown in Fig. 12, and the hyperfine splitting constant was determined only for the Z component of the hyperfine coupling tensor and was found to be 55 gauss.

**DISCUSSION**

The discovery of molybdenum in sulfite oxidase was fortuitous and surprising. The enzyme was examined in the EPR spectrometer for signals which might have been expected from the heme iron, because purified cytochrome b4 exhibits such signals (26). However, the oxidized enzyme gave no detectable EPR signals at the temperature of liquid nitrogen and the enzyme concentration used. Upon reduction, a strong signal with a $g = 1.970$ did appear, whose similarity to previously described molybdenum signals (19, 20) left little doubt as to its significance. Excess dithionite which can reduce molybdenum(V) to lower and diamagnetic valence states eliminated the $g = 1.970$ signal as it had previously been reported to do with other molybdo enzymes (20). Chemical analysis confirmed the presence of molybdenum and demonstrated it to be present in amounts equimolar with the heme. Since most of the molybd enzymes which have been described to date contain flavin, in addition to molybdenum (3-5), the sulfite oxidase was assayed for flavins. Less than 0.045 mole of FAD per mole heme was present, nor was any flavin detected by fluorimetric analysis (15). Furthermore neither FMN nor FAD had any effects on the activities of the enzyme.

That the molybdenum detected in sulfite oxidase was actually a functional part of the enzyme was shown by several lines of evidence. Thus, molybdenum content and enzymatic activity copurified. The molybdenum of sulfite oxidase was reduced by sulfite, but not by xanthine, whereas the molybdenum of xanthine oxidase was reduced by xanthine but not by sulfite. Arsenite which has been shown to inhibit xanthine oxidase and aldehyde oxidase (21) and which is thought to act on the molybdenum of these enzymes (22) also inhibited sulfite oxidase and reduced the intensity of the sulfite-elicited molybdenum(V) signal. The inactivation and the loss of sulfite-reducible molybdenum were coincident and proportional. Cyanide slowly inactivated sulfite oxidase in the presence of sulfite. Cyanide has a high affinity for pentavalent molybdenum (27) and the inactivation caused by cyanide was accompanied by a parallel loss of the sulfite-elicited EPR signal of molybdenum.

The observation that anions inhibited the reduction of ferrocyanide and of cytochrome c by sulfite oxidase, without inhibiting the reduction of oxygen (1), led to the conclusion that the group responsible for transferring electrons from sulfite to the 1-electron acceptors would be reduced in the steady state. The heme prosthetic group of sulfite oxidase was oxidized in the combined presence of sulfite and ferricyanide, whereas the molybdenum was reduced. It may therefore be proposed that the path of electron transfer within sulfite oxidase is

$$\text{Sulfite} \rightarrow \text{Mo} \rightarrow \text{heme} \rightarrow \text{O}_2 \downarrow$$

Ferricyanide or cytochrome c.

We would further stipulate that anions specifically inhibit the step from molybdenum to ferricyanide or cytochrome c. This scheme does not provide any explanation for the observation that the reduction of oxygen to peroxide by this enzyme did not generate detectable univalently reduced oxygen (1).

The power saturation curve for the molybdenum EPR signal of sulfite oxidase is virtually identical with that of the molybdenum signal of xanthine oxidase. The effects of temperature on the EPR signals obtained from enzymatic systems have not been extensively reported although Gibson and Bray have described the effect of temperature on the $g = 1.94$ signal of xanthine oxidase (28). The Mo(V) EPR signals from both sulfite oxidase and xanthine oxidase exhibited a biphase response to changes of temperature. The nonheme iron signal of xanthine oxidase also showed a biphase response to variation of temperature in the range 15$^\circ$K to 130$^\circ$K (28). Presumably, this is diagnostic of a transition in the relaxation modes in the ligand field of the metal, the nature of which cannot now be specified. It has been suggested by Dr. W. Orme-Johnson that this effect could arise from an antiferromagnetic coupling of the molybdenum atom with another paramagnet to yield an EPR-invisible system at the lower temperatures.

The EPR signal of the molybdenum of sulfite oxidase at $-100^\circ$ was 1.6 times more intense than the corresponding signal from the molybdenum of xanthine oxidase. It has been reported that EPR spectrometry detects only 37% of the molybdenum of xanthine oxidase (24). This was based on such EPR standards as Cu$^{++}$-EDTA and pitch. Comparison with a 1 mM Cu$^{++}$-EDTA standard indicated that 50% of the molybdenum in sulfite oxidase was being detected by our EPR techniques. Similarly, the comparison with xanthine oxidase would lead to an estimate of 60% at $-100^\circ$. Increasing the concentration of sulfite 20-fold did not augment the EPR signal obtained from sulfite oxidase, so any failure to detect all of the molybdenum could not have been due to incomplete reduction of Mo(V) due to equilibria.

The EPR signal from sulfite oxidase did not significantly diminish when the enzyme was incubated for 2 hours at room temperature in the presence of 0.005 M sulfite, although there was a gradual loss of signal at longer times of incubation. At $-90^\circ$ the EPR signal of sulfite oxidase was stable for at least 3 days.

Anions, which inhibit the reduction of ferricyanide and of cytochrome c by sulfite oxidase, were, nevertheless, without effect on the EPR spectrum. Changing the pH, however, did change the signal. These changes of signal, when plotted as a function of pH, defined a titration curve which could be characterized by a $pK_a$ of 8.2. This need not reflect the actual $pK_a$ of the ionizable group close to the molybdenum since the pH of the Tris buffers used were measured at 25$^\circ$ whereas the EPR spectra were measured in frozen solutions. It is, nevertheless, clear that a dissociable proton is close enough to the molybdenum to change its EPR spectrum. The splitting effect of this proton could arise from an antiferromagnetic coupling of the molybdenum atom with another paramagnet to yield an EPR-invisible system at the lower temperatures.
field whose parallel and perpendicular components were split by the proton. Removal of this proton converted the signal to that expected from rhombic symmetry around the molybdenum. The size of the proton splitting constants, \( a_{\text{H}}^\text{II} = 10 \) and \( a_{\text{H}}^\text{III} = 12 \), makes it very unlikely that the dissociable proton is bonded directly to the molybdenum (24). It is rather expected that the dissociable proton would be bonded to another atom which was in turn bonded to the molybdenum. Were this other atom nitrogen, it might have exposed its presence by a splitting effect on the Mo(V) signal. Oxygen or sulfur are likely candidates for this other atom. The same conclusion has been expressed for the dissociable group on the molybdenum of xanthine oxidase (25).

During studies of the EPR spectra of xanthine oxidase, Palmer, Bray, and Beinert (19) noted that certain features of the spectrum \((\gamma, \delta)\) were observed at high pH and at very short intervals (milliseconds) after the mixing of enzyme and substrate, whereas other spectral features \((\alpha, \beta)\) were peculiar to low pH and to long periods of exposure of enzyme to substrate (19). These spectral distinctions were ascribed to chemical distinct species of Mo(V) (19). Bray and Vännägard, through the use of D_2O, have recently shown that the \( \alpha, \beta \) signal exhibited features which were due to proton hyperfine splitting of the Mo(V) signal (25). Pisk and Bray concluded that the two forms of molybdenum, which were reflected in the two types of EPR spectra, represented "uncomplexed" molybdenum and molybdenum complexed to substrate (29). We note that the \( \gamma, \delta \) spectrum is very similar to the EPR spectrum of sulfite oxidase seen at high pH, while the \( \alpha, \beta \) spectrum bears resemblance to the low pH spectrum shown in Fig. 9A. This suggests an interpretation of the behavior of xanthine oxidase which differs somewhat from that which has already been advanced (19, 20). Thus the acid and "late" EPR spectrum seems to reflect an axially symmetrical Mo(V), whereas the alkaline and "early" spectrum indicates rhombic symmetry about this molybdenum. It is interesting that, in the case of xanthine oxidase, proton splitting of the Mo(V) spectrum was evident even at pH 10.0 (25). We also observed that in the case of xanthine oxidase, the EPR spectra obtained at pH 7.5 and 10.7 were very similar. It must be noted that all of our measurements were made on solutions frozen a full minute after mixing enzyme and substrate. The lack of a pH effect would indicate that the proton, splitting the Mo(V) signal in xanthine oxidase, exhibits a very high effective pK_a, unlike the corresponding Mo(V) signal. This may be compared to the corresponding values of 18 and 16 gauss obtained with xanthine oxidase (30). At pH 7.0, in water, each of the molybdenum hyperfine peaks of the sulfite oxidase spectrum was split into two by the dissociable proton. At alkaline pH, the hyperfine splitting of the EPR spectrum of sulfite oxidase was difficult to resolve. The hyperfine splitting constant of the \( Z \) component of sulfite oxidase at pD 9.6 was 55 gauss in comparison with the values of 57 gauss (19) or 41 gauss (24) reported for the \( \gamma, \delta \) spectrum of xanthine oxidase.

A somewhat unexpected outcome of the steady state experiment with ferricyanide, evident in Fig. 6, was the finding that the characteristics of the Mo(V) signal in the steady state were different from that seen prior to the addition of ferricyanide. As mentioned earlier, the shape of the Mo(V) signal of sulfite oxidase is markedly dependent on pH (Fig. 9). Thus, while the spectrum in Fig. 6A corresponds to the "basic" Mo(V) spectrum, \( g_m = 1.961 \), the signal in Fig. 6B is almost entirely due to the acid species of Mo(V), with \( g_m = 1.968 \). The spectrum in Fig. 6C, seen after all of the ferricyanide had been reduced, is more acidic than that in Fig. 6A, perhaps owing to the production of considerable amounts of acid during the oxidation of sulfite to sulfate, but more basic than the steady state signal.

Because sulfite oxidase, unlike xanthine oxidase or aldehyde oxidase, has only one paramagnetic center in the reduced state, it would be an excellent candidate for studies of the correlation of the effects of environmental changes on activity and on EPR spectral characteristics. This enzyme has been purified from both chicken liver and human liver by applying the procedure first worked out for the bovine enzyme and in all of these enzymes, reduction with sulfite elicited virtually identical EPR signals.  

Cytochrome \( \alpha \) from microsomes exhibited EPR signals in its oxidized state (26). Our attempts to observe comparable signals in the oxidized state of sulfite oxidase, in the temperature range \(-100^\circ \text{C} \) to \(-196^\circ \text{C}\), have failed. This may be due to the relaxation broadening of the EPR spectrum of this cytochrome (36) and to the relatively low concentrations of sulfite oxidase examined. Hence this point should be reexamined at lower temperatures and at higher concentrations of the enzyme.

Sulfite oxidase is only the third mammalian enzyme shown to contain molybdenum and of the three, it may be the least dispensable. Thus, a genetic defect expressed as an absence of xanthine oxidase has been described in humans and was apparently not seriously disabling (31). Furthermore gout is often treated with allopurinol, which is a potent inhibitor of xanthine oxidase, without serious side effects. Aldehyde oxidase, which is present in rabbit liver, appears to be absent in extracts of dog, pig, steer, sheep, or duck livers (32). Sulfite oxidase, in contrast, appears to be essential since its absence in a human led to severe pathophysiological sequelae (33). It is, thus, an enzyme deserving of further study from several points of view.

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