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HEPATIC SULFITE OXIDASE

IDENTIFICATION OF THE MOLYBDENUM CENTER AS THE SITE OF IRREVERSIBLE INACTIVATION BY FERRICYANIDE

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SUMMARY

Ferricyanide, an artificial electron acceptor for hepatic sulfite oxidase (EC 1 8 3 1), has been shown to cause an irreversible inactivation of the enzyme by an interaction at the molybdenum center The one-electron transferring abilities are lost first, followed by slower parallel losses of oxidase activity and enzymic molybdenum The rate of inactivation by ferricyanide is lower in the presence of cytochrome *c* or any of several anions Accompanying the loss of one-electron transfer activities are changes in the Mo(V) electron paramagnetic resonance signal and in the visible absorption spectrum Specific degradation of the polypeptide chain takes place subsequently, but at a much slower rate than the inactivation These results are discussed in relation to the pathways of electron entry, transport and egress in sulfite oxidase

INTRODUCTION

In the preceding paper [1], the molybdenum center of chicken liver sulfite oxidase was shown to be sensitive to inorganic anions, which caused reversible inhibition of the activity of the enzyme The present paper will show that ferricyanide, an artificial electron acceptor for the enzyme, is capable of causing an irreversible inactivation of sulfite oxidase and will present evidence to implicate the molybdenum center of the enzyme as the site of this inactivation

MATERIALS AND METHODS

Chicken liver sulfite oxidase was purified and assayed as described previously [2] Native rat liver sulfite oxidase and its molybdenum-free apoprotein, purified according to a published procedure [3], were gifts from Dr H J Cohen, National Institute of Environmental Health Sciences, Research Triangle Park, N C , and Dr J L Johnson, of this department, respectively Milk xanthine oxidase was prepared and assayed according to Brady [4]

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Amino acid analyses were performed on a Beckman 120 C amino acid analyzer The content of tryptophan in protein samples was determined by the method of Spande and Witkop [5] Sulfhydryl content was quantitated by the procedure of Ellman [6], as described by Habeeb [7] The method of Forman et al [8] was used for alkylation of proteins with iodoacetate Performic acid oxidation was carried out as described by Hirs [9] The molybdenum content of purified enzymes was measured with a Perkin-Elmer Model 107 atomic absorption spectrometer equipped with a heated graphite furnace (HGA 2000 Controller) Electron paramagnetic resonance (EPR) spectra were recorded as described earlier [2]

Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate was carried out according to the methods outlined previously [2] Proteins used as molecular weight standards and their sources were as follows phosphorylase and carbonic anhydrase (Worthington), transferrin and ovalbumin (Pentex), bovine serum albumin and cytochrome *c* (Sigma), and bovine erythrocyte superoxide dismutase (Truett Labs, Dallas, Texas)

Protein concentrations were determined by the spectrophotometric method of Murphy and Kies [10], or by the method of Lowry et al [11] The former method gave values 10% lower than the latter for the same sample of chicken liver sulfite oxidase

RESULTS

Demonstration of irreversible inactivation of sulfite oxidase by ferricyanide

Earlier studies on chicken liver sulfite oxidase by analytical ultracentrifugation [2] indicated that a dynamic association–dissociation equilibrium existed in solutions of the enzyme An attempt was therefore made to identify the catalytically active species by ultracentrifugal analysis using zone sedimentation through a reaction medium containing sulfite and ferricyanide These experiments required that the enzyme be exposed to concentrations of the electron acceptor higher than those used in the routine assay procedure When sulfite oxidase was incubated with 4 mM ferricyanide at 25 °C in 0 10 M Tris–HCl, pH 8 5, for short periods of time (1–10 min) prior to measurement of activity, extensive inactivation of the enzyme was seen to have occurred Incubation of enzyme alone or enzyme plus sulfite under identical conditions produced no loss of activity

That this inhibition was truly dependent on the presence of ferricyanide and was not reversible by dilution was shown by the following experiment Samples of sulfite oxidase (50 μg/ml) in 0 1 M Tris–HCl, pH 8 5, were incubated at 25 °C with various concentrations of ferricyanide Aliquots of each mixture were withdrawn at intervals for measurement of cytochrome *c* reduction in the presence of sulfite As shown in Fig 1, the time course of inactivation yielded a straight line when plotted on first-order coordinates The pseudo first-order rate constants determined from experiments of this type were dependent on the ferricyanide concentration, the second order rate constant at 25 °C and pH 8 5, obtained by dividing the pseudo-first order rate constants by the ferricyanide concentration used in each case, was calculated to be 250 $\pm$ 30 M^{-1} min^{-1} Appropriate controls demonstrated that the concentrations of ferricyanide carried over into the assay cuvettes were not inhibitory The inhibition was not due to a pH change on the addition of ferricyanide to buffer,

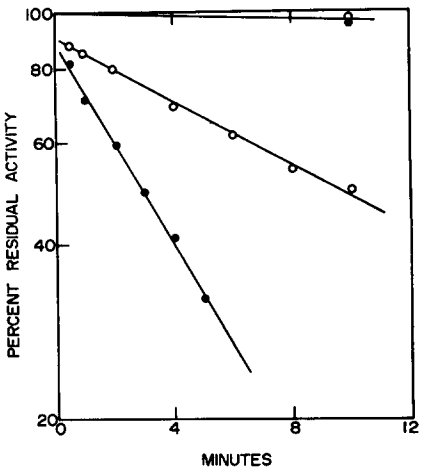


Fig 1 Inactivation of chicken liver sulfite oxidase by ferricyanide Experimental conditions are described in the text The ferricyanide concentrations used were 0 2 mM (○—○) and 0 8 mM (●—●) The top line shows data for control samples incubated at room temperature in the absence of any ferricyanide

and no differences were noted in the rate constant with ferricyanide supplied by different manufacturers or with ferricyanide solutions which were fresh, aged for up to several months, or boiled

Once destroyed by ferricyanide, sulfite oxidase activity could not be restored by dialysis against potassium phosphate, pH 7 8, or by dialysis followed by treatment with excess solid dithionite, ferrocyanide (1 mM), or dithiothreitol (1 mM), for time periods up to several hours

That the effect of ferricyanide on sulfite oxidase is not species-specific was shown by the fact that purified rat liver sulfite oxidase was also irreversibly inhibited, although at a somewhat slower rate Thus, the second order rate constant for inactivation of the rat liver enzyme at 25 °C and pH 8 5 was 40 M^{-1} min^{-1}, the half-time for inactivation by 2 mM ferricyanide was 8 6 min Inactivation of bovine liver sulfite oxidase has also been observed (Cohen H J , personal communication) Milk xanthine oxidase, on the other hand, was not inactivated even by 5 mM ferricyanide, when assayed for its ability to transfer electrons from xanthine to cytochrome *c*, 2,6-dichlorobenzenone-indophenol or ferricyanide

The pH dependence of the inactivation showed a close correspondence to the pH–activity curve, the peak for both occurring at pH 8 5 The dependence of the rate of inactivation on temperature was determined using 0 8 mM ferricyanide at pH 8 5 All assays were done at 25 °C When an Arrhenius plot of the data collected in this manner was constructed, a linear relationship was obtained, and the slope of the line corresponded to an energy of activation of 8 7 kcal By carrying out the incubation with ferricyanide in buffer rendered anaerobic by bubbling with N_2, which had been passed through a column of heated copper powder to remove residual O_2, it was demonstrated that the inactivating effect of ferricyanide did not depend on the presence of O_2

The effect of ferricyanide on sulfite oxidase was shown to be a specific reaction

TABLE I

EFFECTS OF OXIDANTS ON CHICKEN LIVER SULFITE OXIDASE

Oxidant	Concentration (mM)	Time of exposure (min)	Inhibition (%)	Comments
Potassium ferricyanide	0 8	5	65	Independent of O_2, prevented by anions, oxidase activity less sensitive
Methylene blue	0 4	10	70	Dependent on O_2 and light
N-Bromosuccinimide	0 1	5	94	All activities lost in parallel, accompanied by decrease in A_{280} nm, not prevented by anions
H_2O_2	2 0	5	0	
$NaIO_4$	0 8	5	0	
2,6-Dichlorobenzenone-indophenol	0 4	5	4	

occurring at the active site of enzyme by the following experiments First, as shown in Table I, no inhibition of comparable rate or characteristics could be achieved by incubating the enzyme with a variety of other oxidizing agents In addition, significant protection against ferricyanide inhibition was afforded in the presence of inorganic anions, which are presumed to bind at the molybdenum center, by cytochrome *c*, which appears to bind to the enzyme at the same site, and by ferrocyanide These results are presented in Table II It should be pointed out that the protective effect of anions cannot be ascribed to a simple ionic strength effect, since sulfate and phosphate showed significantly different degrees of protection at the same ionic strength The fact that ferrocyanide provides better protection at much lower concen-

TABLE II

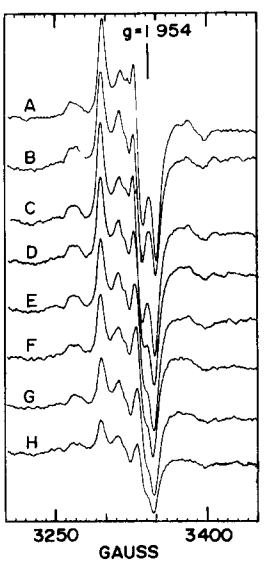
PROTECTION OF SULFITE OXIDASE AGAINST FERRICYANIDE BY ANIONS AND CYTOCHROME *c*

The apparent first-order rate constants for inactivation of sulfite oxidase by ferricyanide are given Enzyme was incubated with indicated concentrations of ferricyanide in 0 1 M Tris–HCl at pH 8 5 The pH was maintained at 8 5 when other additions were made Aliquots were diluted into the assay mixture at various intervals in order to determine the extent of inactivation In all cases incubation mixtures without ferricyanide served as controls

Ferricyanide concentration (mM)	Protective reagent	K_{app}
0 8	—	0 071
0 8	0 2 M Cl^-	0 037
0 8	0 2 M PO_4^{3-}	0 024
0 8	0 2 M SO_4^{2-}	0 012
0 8	5 mM ferrocyanide	0 030
0 8	1 mM cytochrome c^{3+}	0 047
0 2	—	0 025
0 2	1 mM cytochrome c^{3+}	0 012

tration than the other anions tested indicates that ferricyanide inactivation takes place at a site with a specific affinity for the ferricyanide and ferrocyanide ions

When the kinetics of inactivation of the other oxidoreductase activities of sulfite oxidase were examined, it was found that the one-electron transfer reactions (sulfite → cytochrome *c* and sulfite → ferricyanide) disappeared at a somewhat faster rate than the reaction of two-electron transfer to O_2, although both types of activity were eventually totally destroyed These rates could be related to changes in the molybdenum EPR signal of the reduced enzyme, which are illustrated in the series of spectra shown in Fig 2 The first change to be observed was a relatively rapid alteration in shape of the doublet at $g = 1\ 954$ The second slower change resulted in a decrease in the overall signal intensity As shown in Fig 3, the rate of this slower decrease was identical to the rate of loss of oxidase activity The rate of the change in signal shape could not be measured precisely, but it was similar to the rate of loss of cytochrome *c* or ferricyanide reductase activity, both processes being essentially complete by 20 min at 25 °C and pH 8 5 in the presence of 0 4 M ferricyanide



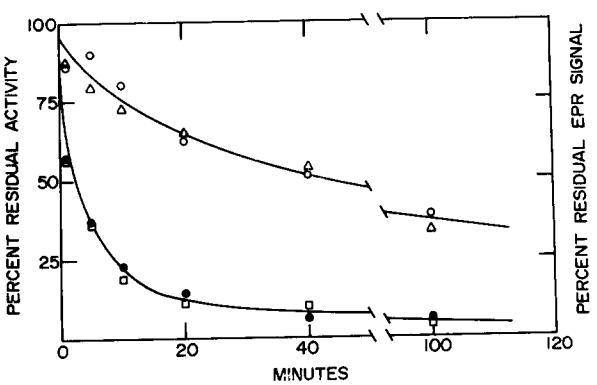


Fig 2 Effect of ferricyanide on the Mo(V) EPR signal of chicken liver sulfite oxidase Purified enzyme (2 mg/ml) was dialyzed versus Tris–HCl, 0 10 M, pH 8 5, and reacted at 25 °C with 0 4 mM ferricyanide Aliquots (200 μl) were removed at various times and added to EPR tubes containing 20 μl 0 10 M sulfite, which served to reduce both the enzyme and the ferricyanide (A) Enzyme removed from reaction vessel before the addition of ferricyanide (B) Ferricyanide added to EPR tube after the addition of ferricyanide to reaction vessel (C) Enzyme removed 1 min after the addition of ferricyanide to reaction vessel (D) 5 min (E) 10 min (F) 20 min (G) 40 min (H) 100 min EPR conditions microwave frequency, 9 140 GHz, microwave power, 5 mW, modulation amplitude, 8 G, time constant, 1 0 s, gain, 3000, temperature, −100 °C, and scan rate, 125 G/min

Fig 3 Comparison of the effects of ferricyanide on the activities and the Mo(V) EPR signal of chicken liver sulfite oxidase The samples from the experiment shown in Fig 2 were examined for their sulfite → cytochrome *c* (●—●), sulfite → ferricyanide (□—□), and sulfite → O_2 (○—○) activities These were plotted, along with the EPR signal amplitudes (△—△), using the sample from Fig 2A as the control in all cases

Several lines of evidence indicated that the progressive decrease in the molybdenum signal amplitude following ferricyanide treatment was indicative of a decrease in the molybdenum content of the enzyme, rather than a lack of reactivity or an apparent change in signal amplitude caused by the changes in line shape First, the size of the signal seen after ferricyanide treatment was not increased by allowing the enzyme to incubate with sulfite for a longer time than usual, prior to freezing in liquid N_2 Second, when a preparation treated with 0 5 mM ferricyanide for 30 min at 25 °C and pH 8 5 was dialyzed against 0 1 M Tris–HCl buffers at different pH and examined by EPR, the typical pH-dependent shift in g value of the molybdenum signal was observed, the shape of the signal at pH 7 was identical to that of native enzyme, while the size was decreased to the same extent as at pH 8 5 Furthermore, double integration of the signal recorded at pH 8 5 after a 20-min exposure to 0 4 mM ferricyanide under the conditions of Fig 3 and comparison with that of untreated enzyme confirmed the fact that approximately 60% of the EPR detectable molybdenum was still present Therefore, the change in signal shape was not introducing an error in the measurements of EPR detectable molybdenum obtained by comparing signal amplitudes Finally, samples of enzyme which had been treated with 1 0 mM ferricyanide for various times were dialyzed, along with a control sample, and the molybdenum content of each was determined by atomic absorption The results of this experiment, illustrated in Fig 4, showed that molybdenum was removed from the protein to the same extent as the loss of the oxidase activity

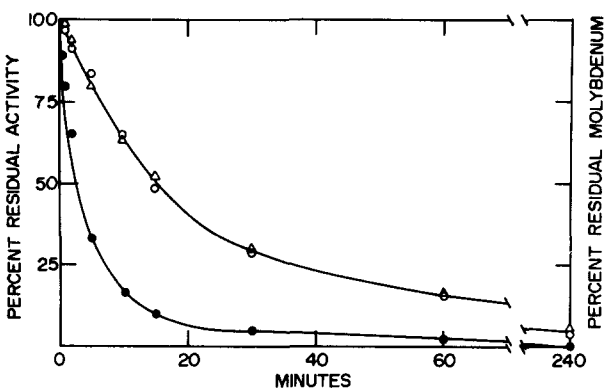


Fig 4 Effects of ferricyanide on the activities and the total molybdenum content of chicken liver sulfite oxidase Aliquots of a solution of purified enzyme (0 66 mg/ml in Tris–HCl, 0 10 M, pH 8 5) were reduced with 5 mM sulfite before and at intervals after the addition of 1 mM ferricyanide All samples were then dialyzed against the same buffer for 18 h and assaayed for enzymatic activity and for molybdenum The triangles represent the molybdenum content, determined by atomic absorption The other symbols are the same as those used in Fig 3

The b_5 cytochrome of sulfite oxidase was unaffected by ferricyanide treatment, as judged by optical absorption spectroscopy During the early stages of ferricyanide inactivation (20 min at 25 °C and pH 8 5 in the presence of 0 4 mM ferricyanide), the heme was still totally reducible by sulfite, although at a somewhat slower rate than that of control enzyme After complete inactivation of the oxidase activity (30 min at 25 °C and pH 8 5 in the presence of 2 mM ferricyanide), sulfite was no longer able

to reduce the cytochrome, while reduction with dithionite was complete and generated an absorption spectrum identical to that of dithionite-reduced native enzyme While the aspects of the visible absorption spectrum attributable to the reduced b_5 cytochrome were unchanged, a difference spectrum (inactive minus native) with a peak at 388 nm was observed in the unreduced enzyme, as shown in Fig 5 The ultraviolet spectrum was unchanged A study of the kinetics of appearance of this difference spectrum, measured as an increase in absorbance at 380 nm, showed that the rate of appearance of the new absorption band was at least as fast as the rate of disappearance of the cytochrome *c* reductase activity

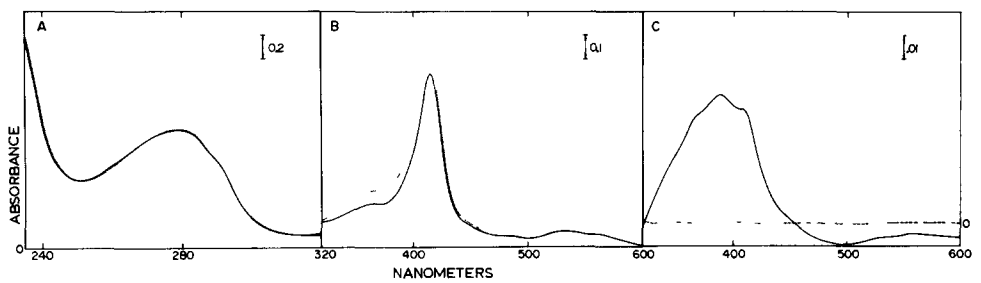


Fig 5 Absorption spectrum of chicken liver sulfite oxidase after ferricyanide inactivation Purified enzyme (2 6 mg/ml) was treated with 1 mM ferricyanide in 0 10 M Tris–HCl, pH 8 5, until the cytochrome *c* reductase activity was destroyed It was then passed through a column (6 cm × 1 cm) of Sephadex G-25 in 0 01 M potassium phosphate, pH 7 8, along with a control sample, and the concentration of each sample was adjusted to 0 5 mg/ml (A) and (B), ultraviolet and visible spectra of control (———) and ferricyanide-treated ( ) enzyme (C) Ferricyanide-treated minus control difference spectrum

Preparations of both chicken and rat liver sulfite oxidase which had been totally inactivated by ferricyanide showed evidence of proteolytic degradation when examined on polyacrylamide gels, both under native conditions and in the presence of sodium dodecylsulfate with and without mercaptoethanol The rate of proteolysis was very much slower than the rate of loss of any of the activities of the enzyme or the rate of loss of molybdenum Thus, one hour after the addition of 1 0 mM ferricyanide, sodium dodecylsulfate gels detected little or no proteolysis, although both the one-electron and two-electron transfer activities of the enzyme were nearly totally abolished

Site of ferricyanide inactivation

The observations that ferricyanide treatment resulted in a loss of molybdenum from the enzyme, that this loss paralleled the loss of oxidase activity, and that anions which markedly influence the molybdenum center [1] protected against ferricyanide inactivation, all made it appear likely that the site of ferricyanide action was at or near the molybdenum prosthetic group However, the apparent proteolysis accompanying ferricyanide treatment, while not occurring on a time scale comparable to the inactivation, suggested that oxidation of a particular amino acid residue at several places in the amino acid chain might offer an alternative explanation Accordingly, an attempt was made to determine whether any amino acid residue was specifically

destroyed after ferricyanide treatment The results of acid hydrolysis (6 M HCl, 110 °C, 24 h) showed no significant differences in the amino acid compositions of control and ferricyanide-inactivated preparations of chicken liver sulfite oxidase Tryptophan content was determined separately by the method of Spande and Witkop [5] and again no difference was found The free sulfhydryl contents of control and ferricyanide-treated enzyme were compared, and a value of 6 moles/mole (40% of the total sulfhydryl groups) was obtained in each case In addition, the fact that higher molecular weight species were not observed when ferricyanide-treated enzyme was studied by sodium dodecylsulfate gel electrophoresis in the absence of mercaptoethanol further substantiated the lack of a primary oxidative effect on the free sulfhydryl groups of the enzyme The possibility of oxidation of methionine was excluded by the fact that no methionine sulfone was detected by acid hydrolysis, the absence of methionine sulfoxide, which cannot by detected by routine acid hydrolysis [12], was confirmed by acid hydrolysis following performic acid oxidation of samples which had been alkylated with iodoacetate, as described by Forman et al [8]

Dietary administration of high levels of tungstate to rats leads to the abolition of sulfite oxidase activity in the tissues [13] and accumulation of inactive, molybdenum-free apoenzyme which can be purified to a state of homogeneity [3] Aposulfite oxidase, purified from livers of tungsten-treated rats, was used to provide further evidence that ferricyanide inactivation takes place at the molybdenum site Thus, while native rat liver enzyme, like that from chicken liver, reacted with 2 mM ferricyanide to produce an increase in absorbance at 380 nm at a rate comparable to the rate of loss of cytochrome *c* reductase activity, reaction of the apo-enzyme under identical conditions produced no change in absorbance The preparation of apoenzyme used in this experiment contained less than 15% of its molybdenum relative to native enzyme, and exhibited 12% residual oxidase activity and 6% residual cytochrome *c* reductase activity The lack of a change in absorbance at 380 nm in the presence of 12% residual oxidase activity appears to be the result of interference by a slow, non-specific reduction of ferricyanide, which is negligible at high concentrations of active enzyme but becomes significant when only a small percentage of the molecules are still reactive

A final piece of evidence that ferricyanide inactivation takes place at the molybdenum center of sulfite oxidase came from studies on the ferricyanide reactivity of enzyme previously exposed to arsenite This agent has been shown, in the case of xanthine oxidase, aldehyde oxidase, and xanthine dehydrogenase, to inhibit reversibly at the molybdenum site and to cause the appearance of a difference spectrum similar to that seen in the present investigation after ferricyanide treatment [13] Cohen et al [14], observed a parallel decrease in sulfite → cytochrome *c* activity, sulfite → O_2 activity, and the sulfite-dependent Mo(V) EPR signal of bovine liver sulfite oxidase after arsenite treatment Studies with the chicken liver enzyme have demonstrated that arsenite is capable of inhibiting the enzyme by a process which initially follows pseudo-first order kinetics, that this inhibition is not reversed by dilution or dialysis, and that enzyme thus inhibited has lost both one-electron and two-electron transfer activities and molybdenum (detected by atomic absorption spectroscopy) to an equal extent No difference spectrum resembling the arsenite spectra of the xanthine-oxidizing enzymes or the ferricyanide spectrum of sulfite oxidase was seen when this enzyme was treated with arsenite However, arsenite was capable of reducing the b_5

cytochrome of the enzyme, and a slow rate of arsenite → cytochrome *c* activity was seen When a preparation which had been inactivated by arsenite (5 mM for 12 h at 25 °C and pH 8 5) was dialyzed, 23% of the molybdenum, 24% of the oxidase activity, and 17% of the cytochrome *c* reductase activity remained Treatment of this sample with ferricyanide (0 4 mM at 25 °C and pH 8 5) revealed almost no reaction as monitored by the absorbance at 380 nm, further demonstrating that the presence of molybdenum at its active site is required in order for ferricyanide to produce its primary spectral effect, and presumably for the later effects as well

The hypothesis that the inactivation of sulfite oxidase by arsenite and ferricyanide take place at a common site was further supported by an experiment which showed that ferrocyanide, in addition to its protective effect against ferricyanide inhibition, was also capable of protecting the enzyme against the effects of arsenite The half-time for inhibition by 2 mM arsenite was 37 min in the absence of ferrocyanide and 82 min in the presence of 2 mM ferrocyanide

DISCUSSION

While ferricyanide has been used for many years as a more or less specific reagent for sulfhydryl groups on proteins [15], and while its ability to oxidize small molecular weight sulfhydryl compounds and cytochromes is well known, the inactivation of sulfite oxidase by ferricyanide, wherein this agent has been shown to act at the molybdenum site to cause an irreversible inhibition, represents an unusual mode of action for this inhibitor The hypothesis that the primary effect of ferricyanide is upon the molybdenum center of sulfite oxidase rests on the following lines of evidence First, the correspondence between the dependence of the rate constant for inactivation and of the enzymatic activity on pH demonstrates a specific effect of ferricyanide related to the catalytic site The small magnitude of the Arrhenius energy of activation further substantiates the existence of a specific process rather than a non-specific effect on the three-dimensional structure of the enzyme, since the latter would be expected to require a very large energy of activation Second, the enzyme is protected against inactivation by agents which are postulated to act at the molybdenum site Third, an early effect on the molybdenum EPR signal of the reduced enzyme can be demonstrated Fourth, this effect is followed by a complete loss of both oxidase activity and molybdenum detectable by EPR or atomic absorption Finally, the early appearance of a difference spectrum strongly resembling that seen following inhibition reactions at the active sites of other molybdenum-containing enzymes [13, 16], as well as the lack of reactivity in this regard of sulfite oxidase from which molybdenum has been removed by in vivo deficiency or by in vitro arsenite treatment, argues in favor of a primary effect at this site None of these studies, however, can completely rule out an effect on a unique active site amino acid residue The inhibition of sulfite oxidase by *N*-bromosuccinimide reported here (Table I) may indicate an interaction between molybdenum and one or more of the amino acid attacked by this reagent at the active site The inhibition seen with *N*-bromosuccinimide shared some of the characteristics of ferricyanide inhibition (irreversibility, partial loss of molybdenum) but was distinguished from ferricyanide inactivation by the fact that both one- and two-electron transferring activities were lost simultaneously, accompanied by a decreased absorbance of the enzyme at 280 nm, and that

anions failed to protect the enzyme from inactivation Although no effect of ferricyanide on the free sulfhydryl content of sulfite oxidase could be detected, the well-known inhibitory effects of sulfhydryl agents on this enzyme [17, 18], and the evidence that molybdenum–sulfur complexes are capable of mimicing the EPR properties of enzymic molybdenum [19–21], lead one to suspect an important role for sulfur at the active site as well

When the original observation of the ferricyanide sensitivity and accompanying degradation of sulfite oxidase was made, it was expected that a specific cleavage at an oxidizable amino acid at selected specific sites of the polypeptide chain would be the primary mechanism of action, especially since previous workers had demonstrated that bovine liver sulfite oxidase was unusually sensitive to the action of trypsin [18] The latter finding, however, could not be reproduced with the chicken liver enzyme (Kessler, D L and Rajagopalan, K V , unpublished) Moreover, the primary cleavage hypothesis is no longer tenable in light of the observations that the cleavage is a very late event in comparison with the loss of enzymatic activity and molybdenum, and that untreated enzyme shows an identical cleavage pattern after prolonged storage There is as yet no explanation for the accelerated proteolytic breakdown of ferricyanide-treated sulfite oxidase However, the fact that reproducibly discrete fragments are formed, one of which contains the cytochrome b_5 with unaltered spectral characteristics, bespeaks of the existence of domains in the quaternary structure of the enzyme

An interesting parallel exists between these results and those of Fukushima et al [22], who found that the high molecular weight b_5 cytochrome isolated from the intermembranous space of mitochondria, which was identified as sulfite oxidase [23], is gradually converted to a small (12 000 dalton) cytochrome b_5 after hypotonic extraction The possible identity of the 11 500 dalton cleavage product of sulfite oxidase with "small hypotonic b_5" of Fukushima et al [22] seems likely since (1) all of the heme of sulfite oxidase retained its b_5-like character following ferricyanide inactivation, and (2) polyacrylamide gels run under native conditions after ferricyanide treatment demonstrated the presence of a new, heme-containing band migrating nearly as fast as the dye band

The data presented above and in the previous article [1] may now be considered in relation of the pathways of electron transport in sulfite oxidase The lack of effect of anions or of ferricyanide treatment on the rate of O_2-dependent oxidation of sulfite shows that the reduction of the molybdenum center by sulfite is not affected under these conditions However, the observation that the line shape of the molybdenum EPR signal is characteristically altered by various anions and in ferricyanide-modified enzyme leads to the conclusion that the susceptible site in both cases is in close proximity to the molybdenum, probably a ligand of the metal It may further be postulated that this ligand is involved in mediating the transfer of electrons derived from sulfite to the one-electron acceptors cytochrome c and ferricyanide but not to O_2 The binding of anions at or near this ligand could cause changes in the symmetry of the molybdenum ligand field as reflected in the EPR spectra [1] and block the transfer of electrons from the molybdenum center to one-electron acceptors Ferricyanide could bind to this site and cause an oxidative modification of the ligand, observable by the increase in absorbance in the region of 380 nm in the difference spectrum of Fig 5, again inhibiting electron transfer to cytochrome c or ferricyanide The modi-

fication by ferricyanide leads to the labilization of the molybdenum center, with the gradual release of the metal from the enzyme, and loss of sulfite $\rightarrow O_2$ activity as well

The identity of the molybdenum ligand remains to be established The lack of difference in the amino acid compositions of native and ferricyanide-treated sulfite oxidase would indicate that the ligand is not one of the common amino acid residues The possibility thus emerges that the coordination field of molybdenum in sulfite oxidase includes one or more non-protein ligands Studies currently in progress on the reconstitution of molybdenum-free sulfite oxidase are expected to shed further light on the likelihood that the molybdenum center of sulfite oxidase includes a low molecular weight cofactor of the type described in the case of nitrate reductase of *Neurospora crassa* [24]

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