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# HEPATIC SULFITE OXIDASE
# EFFECT OF ANIONS ON INTERACTION WITH CYTOCHROME *c*

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## SUMMARY

The one-electron transfer reactions of chicken liver sulfite oxidase (EC 1 8 3 1), with either ferricytochrome *c* or ferricyanide as the electron acceptor, are inhibited by inorganic anions Inhibition by smaller monovalent anions is competitive with respect to cytochrome *c* and mixed non-competitive with respect to $SO_3^{2-}$ Inhibition by larger monovalent anions or by divalent anions is mixed non-competitive with respect to either substrate In contrast, oxidation of $SO_3^{2-}$ in the presence of $O_2$ as electron acceptor is not inhibited by the anions

The electron paramagnetic resonance (EPR) spectrum of Mo(V) in the enzyme is markedly perturbed by the presence of anions, with the change in line shape being characteristic of the anion used These characteristic changes in shape are also seen in the EPR spectrum of sulfite oxidase in mitochondria in the presence of some anions but not others The effects of any two anions on the EPR signal of purified enzyme show a competitive behavior These results demonstrate an interaction of anions with the molybdenum center of sulfite oxidase, which may be the basis of the inhibitory effects

## INTRODUCTION

In 1952 Heimberg and Handler [1] documented evidence suggesting that the conversion of inorganic $SO_3^{2-}$ to $SO_4^{2-}$, the terminal step in sulfur catabolism in higher organisms, must be an enzymatic function Since then the enzyme responsible for this conversion has been studied extensively [2–10] Purified preparations of sulfite oxidase from bovine liver [11] have been shown to contain a $b_5$-type heme [12] and molybdenum [13] as prosthetic groups The same purification procedure has been adapted to yield pure sulfite oxidase from chicken [14], human [14], and rat [15] livers Sulfite oxidase has also been prepared from *Thiobacilli* by Suzuki and coworkers [16, 17] who were unable to identify any prosthetic groups relating to enzyme activity

Abbreviation HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid

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However, evidence for the presence of molybdenum in the bacterial enzyme has since been obtained in this laboratory [14]

The intracellular localization of sulfite oxidase has been the subject of continuing investigation [9, 18], with contradictory findings However, definitive evidence for the localization of the enzyme in the intermembranous space of hepatic mitochondria has been adduced by several groups [19–21] This finding led to the conclusion that cytochrome *c* is in fact the physiological electron acceptor for sulfite oxidase [21] The fact that oxidation of $SO_3^{2-}$ by hepatic mitochondria results in the rotenone-insensitive esterification of phosphate with a $P/SO_3^{2-}$ ratio of 1 corroborates this conclusion [21, 22] Since a natural binding site for cytochrome *c* on the sulfite oxidase molecule is indicated by these findings, it was of interest to probe various aspects of the interaction between the two proteins

The sensitivity of animal and bacterial sulfite oxidase to inhibition by various anions has been described by earlier workers [10, 11, 16, 17] Previous studies on the chicken liver enzyme [14] showed for the first time that anions influence not only the electron-transferring ability, but also the electron paramagnetic resonance (EPR) signal of the molybdenum prosthetic group In the work described below, this observation has been studied in greater detail The results indicate that anions exert their inhibitory action on sulfite oxidase by a specific interaction at or near the molybdenum prosthetic group

## MATERIALS AND METHODS

Cytochrome *c* Type III was purchased from Sigma Purification and assays of chicken liver sulfite oxidase, and equipment and techniques for EPR spectroscopy have been previously described [14] Mitochondria from chicken liver and rat liver were prepared according to a previously published procedure [21] Kinetic analyses and absorption spectra were obtained with a Cary 14 or an Aminco DW-2 recording spectrophotometer, thermostatted at 25 °C Details of the individual experiments are provided in the text

## RESULTS

### *Effects of anions on the activity of sulfite oxidase*

Chicken liver sulfite oxidase was found to be inhibited by various inorganic anions when assayed for its ability to transfer electrons to cytochrome *c* Fig 1 represents an example of experiments of this type, using $Cl^-$ as the inhibiting anion The slight stimulation at low ionic strength seen in Fig 1 was observed with all anions tested The concentrations of the various anions which produced 50% inhibition in this type of experiment are listed in Table I Inhibition by these anions was entirely reversible by dilution Similar results were obtained when ferricyanide was used in place of cytochrome *c* as the electron acceptor However, as reported previously for the bovine enzyme [11], the transfer of electrons to oxygen was not inhibited by anions Indeed, as in the case of the bovine enzyme [11], $SO_4^{2-}$ actually produced a stimulation of the oxidase activity (30% stimulation by 0 04 M $SO_4^{2-}$) This was not a general property of multivalent anions, however, since phosphate was without either an inhibitory or a stimulatory effect on the oxidase activity at pH 8 5 at concentrations as high as 0 5 M

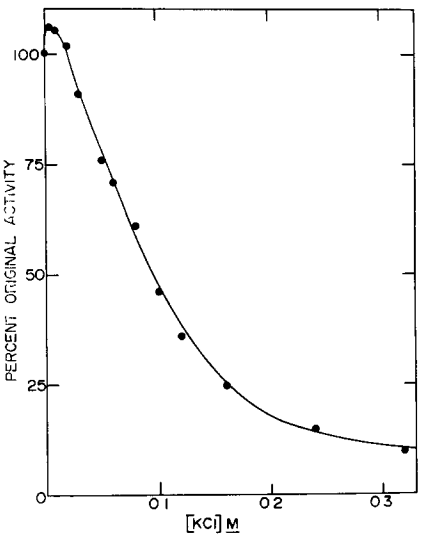


Fig 1 Inhibition of chicken liver sulfite oxidase by KCl Assays were conducted in the presence of KCl in the amounts shown, using the $SO_3^-$ → cytochrome *c* assay Cuvettes contained 0 1 to 0 2 $\mu$g of enzyme, 0 01 mM ferricytochrome *c* and 0 4 mM $Na_2SO_3$ in a final volume of 2 5 ml in 0 005 M Tris–HCl, pH 8 5 Rates were corrected for the slow non-enzymatic reduction of cytochrome *c* by $SO_3^-$

*Effects of anions on the molybdenum EPR signal of sulfite oxidase*

The observation [14] of the interconvertibility of the Mo(V) EPR signal of chicken liver sulfite oxidase between "$Cl^-$" and "phosphate" forms prompted a more thorough investigation of the effects of these and other anions on the signal shape In all cases, characteristic changes in shape were observed at pH 7 at anion concentrations comparable to those tabulated in Table I Fig 2 shows the spectra resulting from the addition of a variety of anions at constant pH to chicken liver sulfite oxidase originally in Tris–HCl, 0 005 M, pH 7 0 Identical results were obtained with sulfite oxidase purified from rat liver

TABLE I

INHIBITION OF SULFITE OXIDASE BY ANIONS

The standard reaction used was the same as in Fig 1 All salts were made up in the same buffer, and the pH was readjusted, if necessary The salt concentrations which inhibited the standard reaction by 50% are indicated

| Salt | Concentration (M) for 50% inhibition |
|---|---|
| NaCl | 0 100 |
| KCl | 0 096 |
| $KNO_2$ | 0 078 |
| KF | 0 072 |
| KNCS | 0 057 |
| $K_2HPO_4$ | 0 026 |
| $K_2SO_4$ | 0 022 |

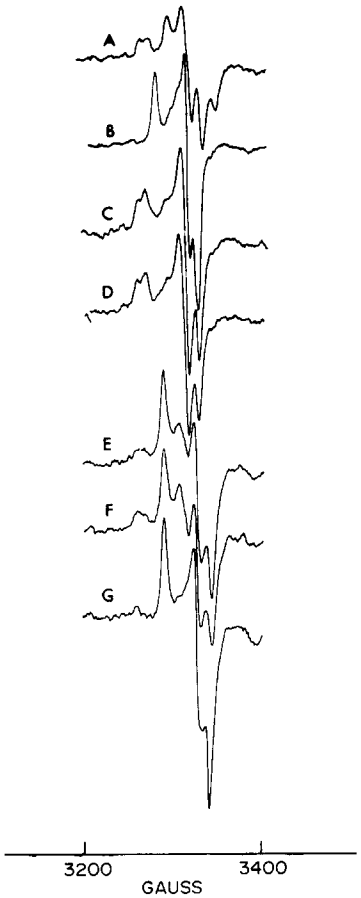


Fig 2 Effects of anions on the Mo(V) EPR signal of chicken liver sulfite oxidase A sample of purified enzyme was dialyzed against Tris–HCl, 0 005 M, pH 7 0 Aliquots of this enzyme solution were reduced with 10 mM $SO_3^{2-}$ and the EPR signal was titrated with $K^+$ salts of various anions The spectra, after completion of the titration in each case, show the influence of A, no anion, B, 0 05 M phosphate, C, 0 10 M $Cl^-$, D, 0 082 M $NO_3^-$, E, 0 053 M $F^-$, F, 0 021 M $SO_4^{2-}$, and G, 0 15 M $CNS^-$ EPR conditions microwave frequency, 9 290 GHz, microwave power, 20 mW, modulation amplitude, 8 G, time constant, 1 0 s, gain 5000, temperature, −100 °C, and scan rate, 100 G/min

The Mo(V) signal of sulfite oxidase undergoes a change from an axially symmetrical to unsymmetrical line shape when the pH is increased from 7 to 9 In contrast to their effects at pH 7, none of the anions tested was able to influence the shape of the Mo(V) EPR signal at pH 9 However, since inhibition of activity is observed at the higher pH, it must be concluded that the failure to affect the signal shape at pH 9 does not reflect lack of binding of the anions to the enzyme

*Kinetics of anion effects*

When the effects of anions on the $SO_3^{2-} \rightarrow$ cytochrome *c* oxidoreductase activity of chicken liver sulfite oxidase were examined kinetically, results such as those depicted in Fig 3 and 4 were obtained In general, smaller monovalent anions were

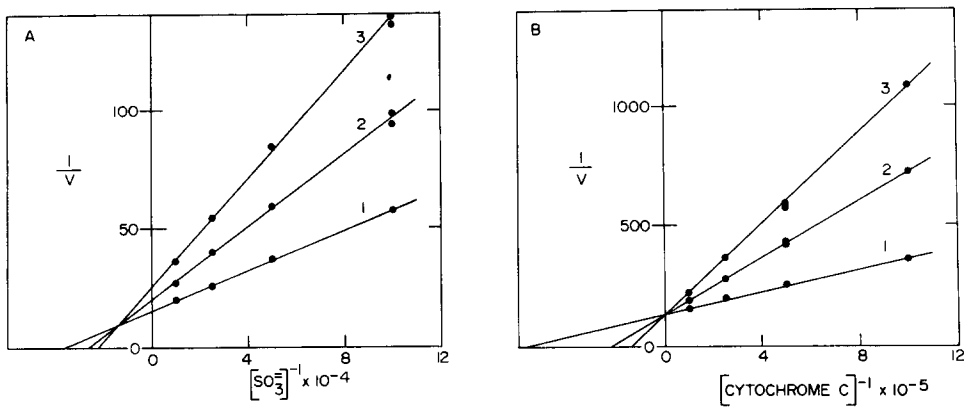


Fig 3 Kinetic analysis of the effect of $Cl^-$ on chicken liver sulfite oxidase All assays were carried out in Tris–HCl, 0 10 M, pH 8 5 A The effect of 1, zero, 2, 0 04 M, and 3, 0 08 M $Cl^-$ on the dependence of activity on $SO_3^{2-}$ concentration at a constant cytochrome *c* concentration of 40 $\mu$M B The effect of 1, zero, 2, 0 02 M, and 3, 0 04 M $Cl^-$ on the dependence of activity on cytochrome *c* concentration at a constant $SO_3^{2-}$ concentration of 0 4 mM Initial velocities ($v$) in all cases are expressed as $\Delta A_{550\ nm}$ per min Duplicate assays which did not agree within $\pm 2\%$ are shown as separate points

mixed non-competitive with respect to $SO_3^{2-}$, but competitive with respect to cytochrome *c*, while larger monovalent anions (with the exception of $NO_3^-$) and multivalent anions displayed mixed non-competitive behavior toward both the donor and acceptor substrates A summary of the inhibition patterns seen, as well as the apparent $K_i$ values determined from plots according to the method of Dixon [23], is provided in Table II Because the 0 1 M Tris–HCl buffer used for all of these experiments con-

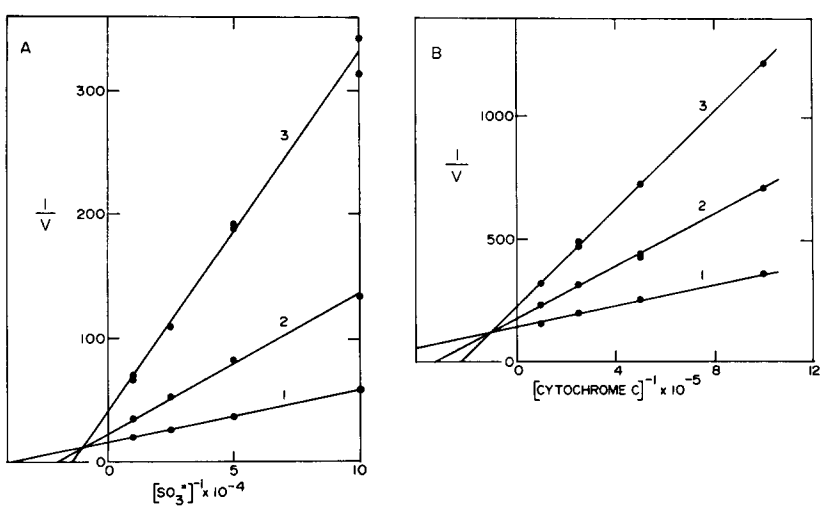


Fig 4 Kinetic analysis of the effect of $SO_4^{2-}$ on chicken liver sulfite oxidase Assay conditions were the same as in Fig 3 A The effect of 1, zero, 2, 0 01 M, and 3, 0 04 M $SO_4^{2-}$ on the dependence of activity on $SO_3^{2-}$ concentration at a constant cytochrome *c* concentration of 40 $\mu$M B The effect of 1, zero, 2, 0 01 M, and 3, 0 02 M $SO_4^{2-}$ on the dependence of activity on cytochrome *c* concentration at a constant $SO_3^{2-}$ concentration of 0 4 mM

TABLE II

PATTERNS OF INHIBITION OF CHICKEN LIVER SULFITE OXIDASE BY VARIOUS ANIONS

The experimental procedures are described in the legend to Fig 3

| Anion | Versus $SO_3^{2-}$ | | Versus cytochrome *c* | |
|---|---|---|---|---|
| | Inhibition type | $K_i(M)$ | Inhibition type | $K_i(M)$ |
| $Cl^-$ | Mixed non-competitive | - | Competitive | - |
| $F^-$ | Mixed non-competitive | 0 102 | Competitive | 0 015 |
| $Br^-$ | Mixed non-competitive | 0 025 | Competitive | 0 017 |
| $NO_3^-$ | Mixed non-competitive | 0 023 | Competitive | 0 013 |
| $I^-$ | Mixed non-competitive | 0 016 | Mixed non-competitive | 0 009 |
| $CNS^-$ | Mixed non-competitive | 0 028 | Mixed non competitive | 0 011 |
| $HPO_4^{2-}$ | Mixed non-competitive | 0 021 | Mixed non-competitive | 0 006 |
| $SO_4^{2-}$ | Mixed non-competitive | 0 008 | Mixed non-competitive | 0 008 |

tained 0 03 M $Cl^-$, it was not possible to calculate meaningful $K_i$ values for this anion

Several factors dictated that the kinetic experiments be performed in 0 10 M buffer, rather than a medium of lower ionic strength Thus, several lots of Type III cytochrome *c* were found to contain an inhibitor of sulfite oxidase whose effects were appreciable in 0 01 M Tris–HCl, pH 8 5, but negligible in 0 10 M buffer at the same pH The inhibitory effect could also be abolished by the addition of either 0 05 M KCl or 0 04% deoxycholate to 0 01 M buffer It seems possible that this inhibition reflects the presence of a polymer of the kind described by Howell and Fridovich [10] Attempts to abolish the inhibition by heating the cytochrome *c* solutions were unsuccessful A second reason for carrying out the kinetic experiments in 0 10 M buffer was the lower rate of $SO_3^{2-}$ auto-oxidation in this medium as compared to 0 01 M Tris–HCl, pH 8 5 Thirdly, the apparent $K_m$ for cytochrome *c* was significantly lower in 0 01 M buffer than in 0 10 M buffer This fact provided confirmation of the competitive behavior of $Cl^-$ toward cytochrome *c*, but it also made experiments in 0 01 M buffer difficult to perform, since rates of absorbance changes at cytochrome *c* concentrations as low as the $K_m$ (approx $10^{-7}$ M) could not be measured without recourse to longer-path-length cuvettes Finally, the use of 0 10 M Tris–HCl eliminated the activation of sulfite oxidase by the lower concentration of anions (see Fig 1) Repetition of the kinetic experiments with $Cl^-$ and $SO_4^{2-}$ versus $SO_3^{2-}$ in 0 01 M Tris–HCl, pH 8 5, produced inhibition plots similar to those shown in Fig 3 and 4, although the apparent $K_i$ was reduced to 0 004 M for $SO_4^{2-}$ Thus, while the inhibition patterns listed in Table II do not appear to be influenced by the ionic strength, the $K_i$ values can be considered valid only for the conditions used

The anions effects on the Mo(V) EPR signal of sulfite oxidase (Fig 2) gave rise to the suspicion that the various anions were acting competitively with each other at a single site Accordingly, precise titrations were carried out for two types of EPR spectral conversions, $Cl^-$ vs $F^-$ and $Cl^-$ vs $SO_4^{2-}$, and in each case the reciprocal of the amplitude of the new negative peak appearing furthest upfield (3337 G) was plotted versus the reciprocal of anion concentration in an attempt to demonstrate such com-

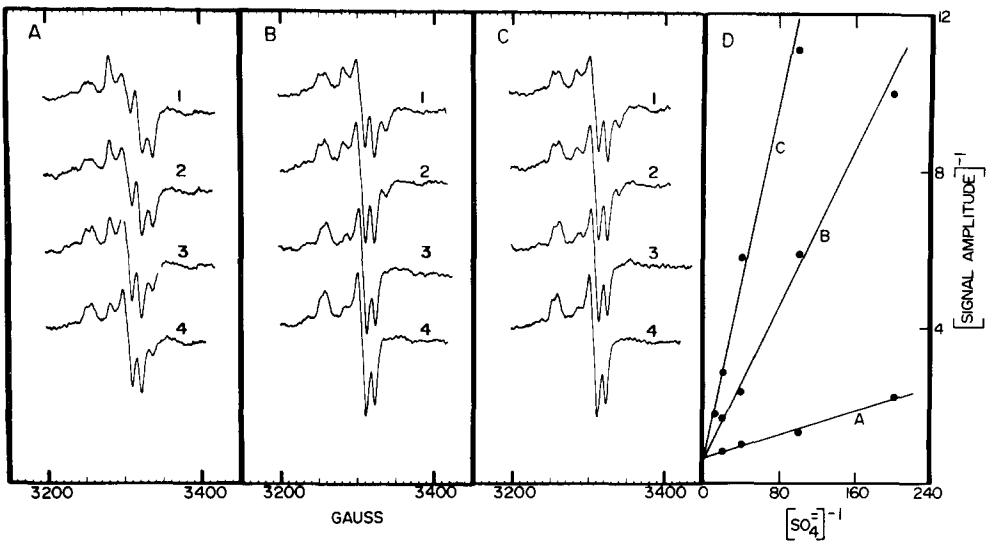


Fig 5 Effect of $SO_4^{2-}$ on the Mo(V) EPR signal of chicken liver sulfite oxidase at various $Cl^-$ concentrations Samples of purified enzyme were dialyzed against A, 0 01 M, B, 0 05 M, and C, 0 10 M Tris–HCl, pH 7 0 Aliquots were dispensed in EPR tubes, the desired amounts of $SO_4^{2-}$ were added, and each sample was reduced with 10 mM $SO_3^{2-}$ 1 min prior to freezing for examination by EPR $SO_4^{2-}$ concentrations used were A, and B 1, 50 mM, 2, 25 mM, 3, 10 mM, and 4, 5 mM, C 1, 83 mM, 2, 50 mM, 3, 25 mM, and 4, 10 mM In D the amplitudes of the peak appearing at 3337 G are plotted on reciprocal coordinates versus the $SO_4^{2-}$ concentrations EPR conditions microwave frequency, 9 143 GHz, microwave power, 5 mW, modulation amplitude, 8 G, time constant, 1 0 s, gain, 5000, temperature, −100 °C, and scan rate, 125 G/min

petition The results of the experiment with $SO_4^{2-}$ are shown in Fig 5 This competitive behavior was also seen with $F^-$

*Effects of anions on sulfite oxidase in its cellular site*

Sulfite oxidase has recently been shown to reside within the intermembranous space of liver mitochondria [19–21], where it is inaccessible to large exogenous electron acceptors, such as cytochrome *c* In its native state, the enzyme reveals a Mo(V) EPR signal in the absence of any added reductant [21, 24] The susceptibility of this "native" signal to the effects of anions was studied and compared to that of the Mo(V) EPR signal of purified enzyme In accord with the results of previously published experiments using whole rat liver homogenates [24], phosphate and $Cl^-$ influenced the Mo(V) EPR signal of the enzyme in its intramitochondrial location, and their effects were identical to those seen with the purified protein, $F^-$ and $SO_4^{2-}$, however, were without effect These results were identical for both chicken and rat liver mitochondria

## DISCUSSION

The results of the above experiments localize the inhibition of sulfite oxidase by inorganic anions, previously observed with the bovine [10, 11] and bacterial [16, 17] enzymes, to the molybdenum center Cohen and Fridovich [11] showed that inhibition

of the bovine enzyme by $SO_4^{2-}$ affected only the one-electron transferring activities, the present results with the chicken liver enzyme extend this observation and show this differential effect to be characteristic of all anions tested Such a differential effect indicates that the inhibited step cannot be the reduction of the enzyme by $SO_3^{2-}$, but rather must be the reduction of the one-electron acceptors by the enzyme It was previously proposed that this reduction of one-electron acceptors takes place at the molybdenum prosthetic group rather than at the $b_5$ cytochrome [12, 13] Hence, the molybdenum moiety was implicated as the site of anion action The present results provide support for the latter suggestion by demonstrating specific effects of anions on the Mo(V) EPR signal of the enzyme, and are compatible with the model for electron-transfer reactions of sulfite oxidase proposed in the accompanying article [25]

The effects of anions on the shape of the Mo(V) EPR signal at pH 7 can be sorted into several different classes For example, $Cl^-$ and $NO_3^-$ generate one shape, while $F^-$ and $SO_4^{2-}$ effect a different shape The phosphate and $CNS^-$ effects represent still other modifications of the EPR spectrum In the absence of more information concerning the environment of the molybdenum atom and the exact binding sites for the anions, it is not immediately obvious why these particular groupings occur None of the EPR spectra contain nuclear hyperfine structures attributable to the interacting anions There is no doubt, however, that whatever their precise binding characteristics, the anions produce definite changes in the symmetry of the molybdenum ligand field, from axially symmetric ($Cl^-$ and $NO_3^-$) to asymmetric (phosphate, $SO_4^{2-}$, and $F^-$) These conclusions are based on the results of experiments in which the anion spectra were generated in $^2H_2O$, as described [14] for $Cl^-$ and phosphate

While it is impossible to say with certainty what shape the molybdenum signal might assume in the absence of any anion, it is probable that the spectrum seen in Fig 2A represents such an anion-free signal, since its shape is different from that seen in Tris–HCl buffers of higher ionic strength or in potassium phosphate buffers after the addition of KCl Moreover, an identical signal was generated in *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES)–KOH, 0 05 M, in which the effects of inorganic anions are eliminated

Despite the separation of anion interactions into distinct classes, the data of Fig 5 indicate that the effects of an ion of one class can be entirely replaced by that of an ion from another class at a high enough concentration, that is, competition appears to be taking place at a common site The conclusion that all anions compete at the one-electron acceptor site is further strengthened by the kinetic results, which indicate competitive behavior toward cytochrome *c* on the part of the smaller anions The lack of a competitive effect by the larger anions does not negate this hypothesis, since it is possible for an inhibitor which competes with both substrates of a two-substrate reaction to give mixed non-competitive inhibition plots versus both substrates [26] In view of the fact that both $SO_4^{2-}$ and $F^-$ are capable of competing successfully with $Cl^-$ in their effect on the Mo(V) EPR signal, although the former gave a mixed non-competitive plot versus cytochrome *c*, while the latter was competitive, the most likely model which can accommodate all of the data is one in which small anions compete only with the electron-accepting substrate while larger anions compete at the electron-donating site as well The juxtaposition of these two sites can be

inferred from previous data [11–13], which demonstrated that only the molybdenum prosthetic group is involved in the transfer of electrons from $SO_3^{2-}$ to the one-electron acceptors The anomalous behaviour of $NO_3^-$, which gave a competitive plot versus cytochrome *c* despite the fact that it is larger than $I^-$, a mixed non-competitive inhibitor, could be a result of steric factors since proper orientation of the flat $NO_3^-$ could allow it to bind selectively to the electron-acceptor site, while the spherical $I^-$ would be large enough, regardless of orientation, to overlap onto the adjacent $SO_3^{2-}$ site

The existence of binding sites for cytochrome *c* (electron acceptor) as well as for $SO_3^{2-}$ (electron donor) in the oxidized form of sulfite oxidase is not inconsistent with the previously described "ping-pong" kinetics displayed by the enzyme The anomalous patterns of product inhibition exhibited by chicken liver xanthine dehydrogenase [27] led to the conclusion that, in enzymes containing internal electron-transport chains, the existence of distinct non-overlapping binding sites for both electron donor and electron acceptor could explain all of the observed kinetic phenomena [28] Northrup [29] observed a similar behavior in the case of biotin-containing transcarboxylase and ascribed it to a "hybrid ping-pong" mechanism which is now recognized more generally [30] The presence of an internal electron transport system comprising molybdenum and heme in sulfite oxidase makes it possible to invoke independent binding sites for $SO_3^{2-}$ and cytochrome *c* in the same form of enzyme This would enable reduced and oxidized forms of cytochrome *c* and various anions to bind to both oxidized and reduced forms of sulfite oxidase

It is now apparent that in liver mitochondria sulfite oxidase is an alternative "reductase" for cytochrome *c* The cytochrome *c* reductase activity of the enzyme is a native property of the enzyme, and becomes observable on release of the enzyme from mitochondria by gentle hypotonic treatment [19–21] The location of sulfite oxidase in the intermembrane space of mitochondria provides ready access to cytochrome *c* located on the outer surface of the inner membrane [31, 32] and readily explains the coupled esterification of phosphate during the oxidation of $SO_3^{2-}$ by mitochondria, with a $P/SO_3^{2-}$ ratio of 1 [21, 22] Thus a study of the interaction of sulfite oxidase and cytochrome *c* is of considerable interest, and could provide further insight into the mechanism of entry of electrons into cytochondria *c* The present studies have implicated an anion-binding site on sulfite oxidase as the site of binding of cytochrome *c* or site of intermolecular transfer of electrons from sulfite oxidase into cytochrome *c* In the future, the use of diverse chemically modified forms of cytochrome *c* as well as of sulfite oxidase is expected to provide further understanding of the interaction of the two molecules

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