Inhibition patterns of a model complex mimicking the reductive half-reaction of sulphite oxidase

Pradeep K. CHAUDHURY, Samar K. DAS and Sabyasachi SARKAR*

Department of Chemistry, Indian Institute of Technology, Kanpur, Kanpur-208016, India

Different inhibition types of the saturation kinetics involving a synthesized model complex, $[\text{Bu}_4\text{N}]_2[\text{Mo}^{\text{VI}}\text{O}_2(\text{mnt})_2]$ (E) (where $\text{mnt}^{2-} = 1,2\text{-dicyanoethylenedithiolate}$), and HSO_3^- as the substrate (S) by structurally similar anions SO_4^{2-} , H_2PO_4^- and H_2PO_3^- have been shown for the first time in relevance to the reductive half reaction of the native enzyme sulphite oxidase. SO_4^{2-} acts as a competitive inhibitor. The mixed-type non-competitive inhibition by H_2PO_4^- and the sigmoidal-type inhibition by H_2PO_3^- are explained by a diamond-configuration random-order model. This involves a random binding sequence of the substrate and the inhibitor, and forms, in addition to two binary complexes [enzyme–substrate (ES) and enzyme–inhibitor (EI)], one enzyme–substrate–inhibitor-type ternary complex (ESI) by participation of at least one more binding site in addition to the catalytic site. This is possible in the present case only by co-ordination enhancement of molybdenum in E. This co-ordination expansion is brought about by nucleophilic attack

of the substrate or the inhibitor at the molybdenum, forming a hepta-coordinated binary complex with the generation of an oxoanionic functional site, called the allosteric site. Analysis of the experimental data suggests that the inhibition by H_2PO_4^- is due to the mechanism following either equilibrium conditions or a combination of steady-state and equilibrium conditions. With H_2PO_3^- , the inhibition is due to the mechanism following the steady-state conditions. It is also shown that the ternary complex involving the enzyme, substrate and H_2PO_4^- or H_2PO_3^- is productive, but at a lower rate than that of the enzyme–substrate binary complex. Mixed-type inhibition with H_2PO_4^- is actually of the type called ‘partially mixed competitive and non-competitive’ as the inhibitor binds both to the catalytic site and to the allosteric site. The sigmoidal-type inhibition by H_2PO_3^- is similar to heterotropic allosteric effect of mixed V,K type with the distinction, however, that the significance of co-operativity in this case is of kinetic importance only.

INTRODUCTION

The pterin-containing molybdenum enzymes catalyse a variety of two-electron reactions with the net exchange of an oxygen atom between substrate and water [1–3]. Among these, the enzyme sulphite oxidase, present in the mitochondrial intermembrane space, catalyses the physiologically vital oxidation of sulphite to sulphate. In this detoxification process the reducing equivalents generated are transferred from sulphite oxidase to ferricytochrome *c* in the respiratory chain, resulting in ATP synthesis [4].

The dimeric enzyme sulphite oxidase contains a molybdenum cofactor (Moco) and cytochrome *b*₅-type haem in each of its two monomeric subunits [5,6]. It has been proposed that the substrate sulphite (or bisulphite) gets oxidized at the molybdenum centre in the reductive half reaction leading to the reduction of the $(\text{Mo}^{\text{VI}}\text{O}_2)^{2+}$ moiety of the Moco by oxygen atom transfer reaction. The reduced Moco, acquiring the $[\text{Mo}^{\text{IV}}\text{O}(\text{H}_2\text{O})]^{2+}$ moiety by co-ordination of water, passes two successive one-electron reducing effects via the *b*₅-type haem to ferricytochrome *c* with the regeneration of the oxidized $(\text{Mo}^{\text{VI}}\text{O}_2)^{2+}$ moiety and releasing two protons in the oxidative half reaction to complete the catalytic cycle [4,7]. The kinetic experiments on this enzyme are further complicated by the inhibitory effects of the anions present in the buffer media used [4]. The inhibition studies on this enzyme with a variety of monovalent and bivalent anions led to the proposition of two-site reactions of independent domains for Moco and ferricytochrome *c*, leading to a hybrid Ping Pong mechanism [4]. In view of the ability of SO_4^{2-} to act as a

competitive antagonist of Cl^- , as is evident from the binding studies, the finding that Cl^- is competitive and SO_4^{2-} mixed non-competitive with respect to ferricytochrome *c* was explained by proposing that SO_4^{2-} is competitive with respect to both substrates, a situation which will generate mixed-type inhibition patterns in steady-state kinetic experiments [4]. The inhibition by phosphate was found to be mixed-type with respect to SO_3^{2-} [4]. The competitive inhibition by SO_4^{2-} is rather expected from the principle of microscopic reversibility since it is the product of the substrate, sulphite. The influence of Cl^- or phosphate on the redox potential data of $\text{Mo}(\text{VI})/\text{Mo}(\text{IV})$ and $\text{Mo}(\text{V})/\text{Mo}(\text{IV})$ couples [8] and on the $\text{Mo}(\text{V})$ EPR signals [9,10] of this enzyme supplement the kinetic findings that these anions directly interact with the substrate binding site of the molybdenum centre of Moco. However, the nature of the anionic binding site and the cause of the different inhibition types of this enzyme are not yet clear [4].

Recently we have shown that the model complex, $[\text{Bu}_4\text{N}]_2[\text{Mo}^{\text{VI}}\text{O}_2(\text{mnt})_2]$ (E) {where $[\text{Bu}_4\text{N}]^+$ is the tetrabutylammonium ion and mnt^{2-} is 1,2-dicyanoethylenedithiolate} follows enzymic substrate (HSO_3^-) saturation behaviour for the oxo-transfer reaction [11] represented by eqn. (1).

Abbreviations used: $[\text{Bu}_4\text{N}]^+$, tetrabutylammonium ion; mnt^{2-} , 1,2-dicyanoethylenedithiolate; E, $[\text{Bu}_4\text{N}]_2[\text{Mo}^{\text{VI}}\text{O}_2(\text{mnt})_2]$; E', $[\text{Bu}_4\text{N}]_2[\text{Mo}^{\text{IV}}\text{O}(\text{mnt})_2]$; ES, enzyme–substrate; EI, enzyme–inhibitor/modifier; ESI, enzyme–substrate–modifier; k_{obs} , observed rate constant; k_{max} , maximum value of the observed rate constant; Moco, molybdenum cofactor.

* To whom correspondence should be addressed.

This complex has the credible co-ordination unit of Moco of sulphite oxidase which is similar to the structurally characterized related tungsten cofactor of the aldehyde ferredoxin oxidoreductase [12]. Electrochemical and EPR studies have shown that chloride anion does bind to E' *cis* to the Mo=O group [11]. This prompted us to investigate the effect of inhibitors on this model reaction in order to understand the nature of the anionic binding site of the native enzyme in terms of structure–function relationships. Inhibition studies on a model reaction in this regard turn out to be promising, since unlike the complexity encountered with the hybrid Ping Pong mechanism of the native enzyme, a model reductive half reaction enables us to study the inhibition mechanism of its reaction pathway, only in a relatively much simpler way. To our knowledge, no such attempt has been made so far using a synthetic analogue of sulphite oxidase. Here we report different inhibition types of this 'abiological' reaction (eqn. 1) by anions such as SO_4^{2-} , H_2PO_4^- and H_2PO_3^- which are structurally similar to the substrate, HSO_3^- . While the observed competitive inhibition by SO_4^{2-} can be understood readily, 'partially mixed competitive and non-competitive' inhibition by H_2PO_4^- and the sigmoid inhibition by H_2PO_3^- have been explained by a 'random-order pathway mechanism', invoking the participation of the catalytic site and the substrate or the inhibitor-induced allosteric site. The sigmoid inhibition is particularly interesting as it demonstrates the ability of this model complex to undergo regulatory behaviour with specific inhibitors.

EXPERIMENTAL

The complex, $[\text{Bu}_4\text{N}]_2[\text{Mo}^{\text{VI}}\text{O}_2(\text{mnt})_2]$, was prepared as described in [11]. AR/GR-grade NaHSO_3 (Merck), $\text{Na}_2\text{SO}_4 \cdot \text{H}_2\text{O}$ (Loba), KH_2PO_4 (BDH) and H_3PO_3 (Aldrich) were used as received. NaH_2PO_3 solution of known strength was made from a dilute solution of H_3PO_3 (2×10^{-2} M) by titrating with NaOH solution (2×10^{-2} M) to adjust the pH to 4.10. The final concentration of the NaH_2PO_3 solution was determined by phosphorus assay following the phosphomolybdc method after oxidation by HNO_3 . From an aliquot of this solution the appropriate strengths were adjusted by dilution.

Kinetic measurements

Reactions were carried out strictly under anaerobic conditions in $\text{CH}_3\text{CN}/\text{water}$ (1:1, v/v) medium and spectrophotometric experiments were performed on a Shimadzu 160 spectrophotometer equipped with a cell compartment thermostatically maintained at 20 ± 0.1 °C. Before kinetic experiments, aqueous solutions of the inhibitors were prepared in combination with the substrate and the solution of the complex was prepared in argon-purged CH_3CN . The rest of the experimental details follow exactly the same procedure as described previously [11]. Since three to four kinetic runs were carried out at each substrate or inhibitor concentration, the mean value of the first-order rate constants and their standard deviations were computed from these. The rate constant values were then fitted to different plots by a conventional curve-fitting procedure (method of least squares).

RESULTS AND DISCUSSION

The results of the inhibition studies with SO_4^{2-} , H_2PO_4^- and H_2PO_3^- as the inhibitors are entered in a tabular form given in Tables 1 and 2.

The competitive inhibition by SO_4^{2-} in the oxidation of HSO_3^- to HSO_4^- by a synthesized low-molecular-mass compound is, to our knowledge, the first model behaviour similar to a text-book

example of an enzymic competitive inhibition. This kinetic evidence directly supports the proposed competitive inhibition by SO_4^{2-} with respect to both sulphite and cytochrome *c* to rationalize the observed mixed non-competitive inhibition by SO_4^{2-} and is in favour of the existence of two separate and independent binding sites at Moco and ferricytochrome *c*, leading to a hybrid Ping Pong mechanism [4]. The inhibition of native enzyme by the phosphate ion is reported to be mixed non-competitive [4]. Therefore, a similar interpretation as is given for SO_4^{2-} inhibition could also account for this. However, in all probability this may not be so, as is suggested by the results of H_2PO_4^- inhibition in the present study. The mixed non-competitive inhibition by H_2PO_4^- suggests a two-site interaction of the inhibitor with the model complex. Bray has shown [13] that the ^{17}O -enriched phosphate-inhibited native enzyme exhibits a characteristic Mo(V) EPR spectrum with hyperfine lines due to the coupling of the ^{17}O nucleus, which is consistent with bidentate co-ordination of phosphate at the Mo(V) centre. Sulphite oxidase, known to be present in the mitochondrial intermembrane space, catalyses sulphite oxidation coupled to one equivalent of

Table 1 Primary plots

Inhibitor	Plot	Nature
SO_4^{2-}	$1/k_{\text{obs}}$ versus $1/[\text{S}]$ at different fixed concentrations of the inhibitor (Figure 1)	Typical of competitive inhibition with $K_i = 9.6 \times 10^{-3}$ M, $k_{\text{max}} = 0.87 \text{ s}^{-1}$
H_2PO_4^-	$1/k_{\text{obs}}$ versus $1/[\text{S}]$ at different fixed concentrations of the inhibitor (Figure 2)	Typical of mixed non-competitive inhibition
H_2PO_3^- *	(a) k_{obs} versus $[\text{S}]$ at different fixed concentrations of the inhibitor (Figure 3) (b) the corresponding double-reciprocal plots (Figure 3, inset)	(a) Sigmoid; the sigmoidicity increased, with the decrease in k_{max} on increasing the concentration of H_2PO_3^- (b) Concave upwards

* H_2PO_3^- , which is a potent reducing agent, was found to be kinetically very slow, being almost insignificant as compared with HSO_3^- towards the complex.

Table 2 Secondary plots

Inhibitor	Plot	Nature
H_2PO_4^-	(a) k_{obs} versus H_2PO_4^- at a fixed non-saturating concentration of the substrate (Figure 4) (b) $1/k_{\text{obs}}$ versus H_2PO_4^- concentration (Figure 7) or the slope replot (Figure 8) (c) Intercept replot (Figure 7)	(a) Hyperbolic, curving downwards and levelling off at a finite value (b) Hyperbolic, curving upwards (c) Hyperbolic, curving upwards
H_2PO_3^-	(a) k_{obs} versus H_2PO_3^- concentration at a fixed non-saturating concentration of the substrate (Figure 5) (b) k_{obs} versus H_2PO_3^- concentration at a fixed saturating concentration of the substrate (Figure 6)	(a) One of the four possible curve types characteristic of a second-degree rate equation in inhibitor concentration (b) Hyperbolic, curving downwards

Figure 1 Competitive inhibition of SO_4^{2-} , shown for two different fixed concentrations of SO_4^{2-} (I_1 and I_2) along with the double-reciprocal plot of the substrate saturation kinetics ($I = 0$)

ATP synthesis [4]. The mixed non-competitive inhibition by phosphate suggests the regulatory behaviour of this enzyme is relevant to the main oxidative phosphorylation reaction. With H_2PO_3^- the saturation curve was sigmoidal with $K_{0.5} = 4.9 \times 10^{-2} \text{ M}$ and the observed $k_{\text{max}} = 0.53 \text{ s}^{-1}$. The sigmoidicity increased ($K_{0.5} = 8.40 \times 10^{-2} \text{ M}$) with the decrease in observed k_{max} (0.30 s^{-1}) on increasing the concentration of H_2PO_3^- as shown in Figure 3. The corresponding double-reciprocal plots (Figure 3, inset) are typical of positive co-operativity [14]. However, it is well known that sigmoidism in the case of a monomeric enzyme in combination with a modifier, particularly when the enzyme has a single substrate binding site, cannot be due to a co-operative model [14], nor can it be due to a kinetic model involving an ordered pathway mechanism [15], but it can arise if the reaction follows a branched-pathway-type mechanism (random-order model) under the steady-state conditions [15–25]. In a typical random-order model, the substrate (S) and the modifier (I) bind at two points in the reaction sequence and form, in addition to two binary complexes (ES and EI), one enzyme–substrate–modifier (ESI)-type ternary complex. This is known as the diamond-configuration random-order model [26] as shown in Scheme 1.

The steady-state rate function for this model, either in modifier or in substrate concentration at a fixed non-saturating concentration of the other, is second degree and hence can assume a rate-dependence pattern that can be one of the ten possible curve types characteristic of a second-degree equation [19]. The rate function, however, becomes hyperbolic at the fixed saturating concentration of either the substrate or the inhibitor [19]. In a more complex branched-pathway-type mechanism, the modifier

or the substrate can bind at more than two points in the reaction sequence and as a result the degree of the steady-state rate function, either in substrate or in modifier concentration, is greater than two and generally shows a more complex type of rate-dependence pattern [20,21,24]. Elucidation of the structure of the kinetic pathways of such types of models is possible by exploitation of the fact that under the steady-state conditions the degree of the rate function, either in substrate or in modifier concentration at a fixed non-saturating concentration of the other, represents the number of times the substrate or the modifier binds with the enzyme forms in the overall reaction sequence [20,25]. On the other hand, if the model under consideration follows quasi-equilibrium conditions, the degree of the rate function, either in substrate or in modifier concentration, is less than the number of times the substrate or the modifier binds with the enzyme forms [25]. Saturating concentrations of either the substrate or the modifier do not reduce the degree of the rate function with respect to each other. This helps us differentiate between the steady-state and the quasi-equilibrium kinetics of a selected model. Further it is also possible that a random-order model can operate under the combined influence of both steady-state and quasi-equilibrium conditions valid in different parts of the reaction pathways network [27–29]. Double-reciprocal plots constitute, therefore, a part of the type of investigation mentioned above with respect to the substrate. A more or less complete idea regarding the structure of the kinetic pathways is possible by further investigation with regard to the degree of the rate function as a function of the modifier concentration. This, in the case of linear double-reciprocal plots, is usually represented by an inhibition plot (k_{obs}^{-1} versus $[I]$). An alternative to this is to analyse the slope and the intercept replot of the double-reciprocal plots. Such analyses are of valuable assistance in elucidating the structure of the kinetic pathways of a model [30,30a]. Since the diamond-configuration random-order model, apart from under its equilibrium conditions, can lead to simpler inhibition types under steady-state conditions also [17,31–33], it is of further interest here in view of the mixed-type inhibition by H_2PO_4^- and the sigmoidal-type inhibition by H_2PO_3^- . Although this model in the present context requires attention, it must be emphasized that in the case of a model complex responding only to the reductive half reaction of the native enzyme, the model complex, unlike the real enzyme, is not regenerated as the product is formed. In view of this, Scheme 1 is modified to Scheme 2. As a consequence the coefficients of the steady-state rate expression [eqn. (A1), Appendix] differ from that given by the method of King and Altman [34] applicable to real enzymes. Similarly the conditions which give rise to mixed non-competitive inhibition are also not exactly the same as in the case of a real enzyme [17,18,32,33]. This is inferred by arriving at the rate expressions for Scheme 2 under different kinetic conditions (see the Appendix). The conditions which give rise to mixed non-competitive inhibition are: (i) E, ES, EI and ESI are in equi-

Scheme 1

Scheme 2

Figure 2 Partially mixed competitive-non-competitive inhibition by KH_2PO_4

Double-reciprocal plot without inhibitor ($[I] = 0$) is also shown.

Figure 3 (a) Hyperbolic plot of substrate saturation kinetics and sigmoidal-type rate dependence (b) with 20×10^{-4} M NaH_2PO_3 and (c) with 50×10^{-4} M NaH_2PO_3

Inset, the corresponding double-reciprocal plots.

librium; and (ii) E, ES and EI are in equilibrium, but the steady-state condition applies to ESI.

Prior to considering Scheme 2 it is necessary to ascertain that the different inhibition types are not due to the participation of H^+ as a moderator in Scheme 2. This becomes possible by analysing the role of pH in reaction (1) both in the absence and in the presence of the inhibitor. Reaction (1) was found to be accompanied by a drop in pH of the medium from 5.5 to 4.5. This is due to a large difference in $\text{p}K_a$ values of HSO_3^- (7.25) and of the corresponding product HSO_4^- (1.92). On the other hand reaction (1) is pH-dependent also. The reaction rate increased on decreasing the initial pH by addition of acetic acid. Hence the kinetic data fitting a first-order plot over a larger initial fraction of time at first seem to be conflicting, but this is due to the buffering action by the protic equilibrium mixture of the free and possibly of the bound substrate species [11]. The extent to which the reaction proceeds without the variation of pH depends on the concentration of HSO_4^- formed before it exceeds

Figure 4 Observed rate (k_{obs}) variation with KH_2PO_4 concentration at a fixed non-saturating concentration of NaHSO_3 (0.02 M)

the buffer limit. An experimental verification of this fact came from reaction (1) with half the concentration of E (2×10^{-4} M) when there was no noticeable pH change. The Michaelian-type behaviour of reaction (1) reported earlier [11] rules out the possibility of H^+ acting as an inhibitor [14,23] in Scheme 2 under the combined assumption of steady-state and equilibrium conditions. This is because a sigmoidal saturation curve is predicted with the prior knowledge that E and ES are in equilibrium [11] and assuming the rest of the steps follow the steady-state conditions. Different inhibition types with H_2PO_4^- and H_2PO_3^- rule out the participation of H^+ in Scheme 2 under equilibrium conditions also. This is simply because the $\text{p}K_a$ values [35] of these two inhibitors are nearly the same as that of HSO_3^- , and hence the pH change observed at the concentrations used on completion of the reaction was also the same as was found without the use of inhibitors. Different inhibition types under identical pH conditions can not therefore be due to the participation of H^+ .

In light of the random-order model (Scheme 2) the sigmoidal data (Figure 3) indicate the binding of the substrate at two points in the reaction sequence. To know the binding sequence of the inhibitor in the reaction pathways, the rate-dependence characteristics with the various inhibitor concentrations at a fixed non-saturating and saturating concentration of the substrate were investigated for both H_2PO_4^- and H_2PO_3^- . Figures 4 and 5 represent data for H_2PO_4^- and H_2PO_3^- respectively at the fixed non-saturating concentration of the substrate. The levelling off of the rate function at a finite value with increasing concentrations of H_2PO_4^- and H_2PO_3^- is indicative of the productive nature of the ternary complex [14]. With H_2PO_3^- the inhibition type (Figure 5) is identified to be one of the four possible curves types [19] characteristic of a second-degree rate equation in inhibitor concentration at a fixed non-saturating concentration of the substrate, which becomes hyperbolic at the saturating concentration of the substrate (Figure 6). This implies the binding of H_2PO_3^- at two points in the reaction sequence while the reaction overall follows the steady-state conditions [eqn. (A2), Appendix]. With H_2PO_4^- the inhibition plot (k_{obs}^{-1} versus $[I]$) was found to be hyperbolic (Figure 7) at the fixed non-saturating concentration of the substrate and this was substantiated by the hyperbolic nature of the slope replot (Figure 8). These results are consistent

Figure 5 Observed rate (k_{obs}) versus NaH_2PO_3 concentration at a fixed non-saturating concentration of NaHSO_3 (0.025 M)

Figure 6 (a) Hyperbolic plot of observed k_{max} versus NaH_2PO_3 at the fixed saturating concentration of NaHSO_3 (0.18 M) and (b) the corresponding double-reciprocal plot

with the productive nature of the ternary complex. The hyperbolic nature of the intercept replot (Figure 7) represents the behaviour of the rate function at the saturating concentration of the substrate. The rate constant for the breakdown of the ternary complex into the product, k_4 , was obtained separately in the case of both the inhibitors from $k_{\text{obs}}/[\text{HSO}_3^-]$ versus k_{obs} plot [36] (results not shown) at a fixed saturating concentration of the

Figure 7 Reciprocal of observed rate (k_{obs}^{-1}) versus KH_2PO_4 concentration at fixed (I) 0.01 M HSO_3^- , (II) 0.02 M HSO_3^- and (III) the y -intercept of the reciprocal plot (Figure 2) versus KH_2PO_4 concentration

Figure 8 Hyperbolic dependence of slope (Figure 2) with the variation of KH_2PO_4 concentration

The data points with 0.025 M KH_2PO_4 are not shown in Figure 2 for clarity.

respective inhibitor. The values of k_4 thus obtained for H_2PO_4^- and H_2PO_3^- were 0.620 s^{-1} and 0.006 s^{-1} respectively. For H_2PO_4^- , the inhibition constants $K_1 = 2.69 \times 10^{-3} \text{ M}$ and $K_1' = 8.15 \times 10^{-3} \text{ M}$ together with $k_4 = 0.635 \text{ s}^{-1}$ were evaluated from the reciprocal plot [14] (Figure 2) and their values were also checked by an alternative plot introduced by Yoshino [37], using the known value of $K_s = 1.00 \times 10^{-2} \text{ M}$. The value of k_4 in the case of H_2PO_4^- , as obtained by two independent means, appears to be in good agreement within experimental error. Apart from the diamond-configuration random-order model with partial inhibition (ESI being productive), non-linear inhibition plots are also possible for other branched-pathway-type models wherein the inhibitor can bind twice to the same form of the enzyme [14]. A discrimination, however, is possible from the nature of the slope and the intercept replot, which in the present case rules out the latter possibility. Therefore these results point towards the validity of the diamond-configuration random-order model either under equilibrium conditions [eqn. (A4), appendix] or under the combined assumption of steady-state and equilibrium conditions [eqn (A3), Appendix]. In either case the substrate or the inhibitor binds at two points in the reaction sequence. A further distinction between the two, however, is not possible by kinetic studies

alone. In view of this the significance of K_1' should be treated as operational. The kinetic pathways of the reaction with H_2PO_4^- or H_2PO_3^- as the inhibitor have been diagnosed to be as shown in Scheme 2. At this stage we propose that the anionic binding of the substrate (S) or the inhibitor (I) transforms the hexa-coordinated complex containing the $(\text{Mo}^{\text{VI}}\text{O}_2)^{2+}$ moiety into a hepta-co-ordinated (ES)- or (EI)-type intermediate with the generation of an oxoanionic allosteric site in a manner implicit in Koshland's induced fit theory [38]. The participation of these two sites in the reaction actually forms the basis of the random-order mechanism (Scheme 2).

It follows, therefore, that the mixed non-competitive inhibition is actually of the type called 'partially mixed competitive and non-competitive' as the inhibitor binds both to the catalytic site and to the allosteric site [39] and the ternary complex ESI is productive. The sigmoidal inhibition in the case of a model complex is the first of its kind to be found and is similar to heterotropic allosteric inhibition [14] of mixed V,K-type [14,40], with the only distinction being that the significance of cooperativity in this case is of kinetic importance only [41,42]. Since inhibition of sulphite oxidase by H_2PO_3^- is not known, it will be interesting to verify this in the light of the present model study.

Hence the validity of the random-order pathway mechanism in the case of a monomeric model compound has been observed for the first time, as far as known, with real enzymes. This enhances our understanding of the oxotransfer mechanism of the native enzyme in the presence of a modifier, and also explains the regulatory behaviour of monomeric model compounds in general. Furthermore, the proton-involved oxotransfer model reaction presented here or earlier unambiguously establishes that HSO_3^- acts as a substrate; however, it still remains to be seen whether SO_3^{2-} is also a substrate in the said reaction.

This work was supported by the Department of Science and Technology, New Delhi, Government of India.

REFERENCES

- Enemark, J.H. and Young, C. G. (1993) *Adv. Inorg. Chem.* **40**, 11–88
- Rajagopalan, K. V. (1991) in *Advances in Enzymology and Related Areas of Molecular Biology* (Meister, A., ed.), vol. 64, pp. 215–290, John Wiley & Sons, Inc., New York
- Pilato, R. S. and Stiefel, E. I. (1993) in *Inorganic Catalysis* (Reedijk, J., ed.), pp. 131–188, Marcel Dekker, Inc., New York
- Rajagopalan, K. V. (1980) in *Molybdenum and Molybdenum-containing Enzymes* (Coughlan, M. P., ed.), pp. 241–272, Pergamon Press, Oxford
- Cohen, H. J. and Fridovich, I. (1971) *J. Biol. Chem.* **246**, 367–373
- Cohen, H. J., Fridovich, I. and Rajagopalan, K. V. (1971) *J. Biol. Chem.* **246**, 374–382
- Hille, R. and Massey, V. (1985) in *Molybdenum Enzymes* (Sprio, T. G., ed.), pp. 443–518, Wiley Interscience, New York
- Spence, J. T., Kipke, C. A., Enemark, J. H. and Saunde, R. A. (1991) *Inorg. Chem.* **30**, 3011–3015
- Bray, R. C., Gutteridge, S., Larry, M. T. and Wilkinson, T. (1983) *Biochem. J.* **211**, 227–236
- Kessler, D. L. and Rajagopalan, K. V. (1972) *J. Biol. Chem.* **247**, 6566–6573
- Das, S. K., Chaudhury, P. K., Biswas, D. and Sarkar, S. (1994) *J. Am. Chem. Soc.* **116**, 9061–9070
- Chan, M. K., Mukund, S., Kletzin, A., Adams, M. W. W. and Rees, D. C. (1995) *Science* **267**, 1463–1469
- Bray, R. C. (1986) *Polyhedron* **5**, 591–595
- Dixon, M. and Webb, E. C. (1979) in *Enzymes*, 4th edn., pp. 401, 441, 446, 341, 358, 345, 399–400, 440, Longman, London (This order of page citations corresponds to the citations of this reference in the text.)
- Sweeny, J. R. and Fisher, J. R. (1968) *Biochemistry* **7**, 561–565
- Ingraham, L. and Makower, B. (1954) *J. Phys. Chem.* **58**, 266–270
- Botts, J. and Morales, M. (1955) *Trans. Faraday Soc.* **49**, 696–707
- Laidler, K. J. (1956) *Trans. Faraday Soc.* **52**, 1374–1382
- Botts, J. (1958) *Trans. Faraday Soc.* **54**, 593–604
- Wong, J. T.-F. and Hanes, C. S. (1962) *Can. J. Biochem. Physiol.* **40**, 763–804
- Griffin, C. C. and Brand, L. (1968) *Arch. Biochem. Biophys.* **126**, 856–863
- Ferdinand, W. (1966) *Biochem. J.* **98**, 278–283
- Laidler, K. J. (1955) *Trans. Faraday Soc.* **51**, 528–539
- Bardsley, W. G. (1976) *Biochem. J.* **153**, 101–117
- Wong, J. T.-F. and Endrenyi, L. (1971) *Can. J. Biochem.* **49**, 568–580
- Hanes, C. S., Bronskill, P. M., Gurr, P. A. and Wong, J. T.-F. (1972) *Can. J. Biochem.* **50**, 1385–1413
- Cha, S. (1968) *J. Biol. Chem.* **243**, 820–825
- Frieden, C. (1970) *J. Biol. Chem.* **245**, 5788–5799
- Ainslie, G. R., Shill, J. P. and Neet, K. E. (1972) *J. Biol. Chem.* **247**, 7088–7096
- Cleland, W. W. (1963) *Biochim. Biophys. Acta* **67**, 104–137
- Cleland, W. W. (1963) *Biochim. Biophys. Acta*, **67**, 173–187
- Frieden, C. (1964) *J. Biol. Chem.* **239**, 3522–3531
- Hearon, J. Z., Bernhard, S. A., Fries, S. L., Botts, B. J. and Morales, M. F. (1959) in *The Enzymes*, 2nd edn., vol. 1, (Boyer, P. D., Lardy, H. and Myrback, K., eds.), pp. 49–142, Academic Press, New York
- Morales, M. (1955) *J. Am. Chem. Soc.* **77**, 4169–4170
- King, E. L. and Altman, C. (1956) *J. Phys. Chem.* **60**, 1375–1378
- Cotton, F. A. and Wilkinson, G. (1988) in *Advanced Inorganic Chemistry*, 5th edn., p. 105, Wiley Interscience, New York
- Laidler, K. J. (1958) in *Chemical Kinetics of Enzyme Action*, pp. 86–87, Oxford University Press, Oxford
- Yoshino, M. (1987) *Biochem. J.* **248**, 815–820
- Koshland, Jr., D. E. (1958) *Proc. Natl. Acad. Sci. U.S.A.* **44**, 98–104
- Krupka, R. M. and Laidler, K. J. (1961) *J. Am. Chem. Soc.* **83**, 1445–1447
- Changeux, J.-P. (1964) *Brookhaven Symp. Biol.* **17**, 232–249
- Sanwal, B. D. and Cook, R. A. (1966) *Biochemistry* **5**, 886–894
- Whitehead, E. (1970) *Prog. Biophys. Mol. Biol.* **21**, 321–396

APPENDIX

The derivation of the rate expressions under different kinetic conditions

(a) Steady-state conditions

This situation arises when the product release steps in Scheme 2 (main paper) are much faster compared with the other steps in the network. As a result the concentrations of the enzyme species, [ES], [EI] and [ESI], are away from equilibrium.

k_{obs} is then given by the following expression (eqn. A1):

$$k_{\text{obs}} = \frac{\{k_3 k_1 k_{-2} l [S] + k_3 (k_1 k_1' l - k_1 k_{-1}' k_1') [S]^2 + (k_3 k_2 k_{-2}' k_1' + k_4 k_1 k_{-2} k_2' + k_4 m k_1' k_2') [S][I] + k_4 k_1 k_1' k_2' [S]^2 [I] + k_4 k_2 k_1' k_2' [S][I]^2\}}{\{l m k_{-2} + (l m k_1' - m k_1' k_{-1}' + k_1 k_2 l) [S] + (l k_{-2} k_2' - k_2' k_{-2}' k_{-2} + m l k_2) [I] + (l k_1' k_2' - k_1' k_{-1}' k_2' - k_2' k_{-2}' k_1' + k_1 k_{-1}' k_2' + k_2 k_{-2}' k_1' + k_1 k_2' k_{-2} + m k_1' k_2') [S][I] - k_1' k_{-1}' k_1 [S]^2 + k_2 k_2' l [I]^2 + k_1 k_2' k_1' [S]^2 [I] + k_2 k_1' k_2' [S][I]^2\}} \quad (\text{A1})$$

where $l = k_{-1}' + k_{-2}' + k_4$ and $m = k_{-1} + k_3$.

Since reaction (A1) follows quasi-equilibrium conditions (reference [11] of the main paper), $k_3 \ll k_{-1}$; the rate coefficients in eqn. (A1) modify but its form remains unchanged.

Eqn. (A1) in a simpler form can be represented as:

$$k_{\text{obs}} = (a[S] + b[S]^2 + c[S][I] + d[S]^2[I] + e[S][I]^2) / (\text{const.} + p[S] + q[I] + r[S][I] + t[S]^2 + u[I]^2 + v[I]^2[S] + w[S]^2[I]) \quad (\text{A2})$$

where a, b, c, d and p, q, r , etc. are combinations of rate constants. The rate expression is second degree both in substrate and inhibitor concentration at the fixed non-saturating concentration of the other, but it becomes hyperbolic when the concentration of either of the two species is fixed at the saturating level.

(b) Combined assumption of steady-state and equilibrium conditions

In Scheme 2 of the main paper, when E, ES, EI are in equilibrium but ESI is at the steady state the expression for k_{obs} then comes out to be (eqn. A3)

$$(k_3 + k_4[I]/K_1') / \{ (1 + [I]/K_1') + K_s/[S](1 + [I]/K_1') \} \quad (\text{A3})$$

where $1/K_1' = (k_1'K_s + k_2'K_1)/KK_1$, with $K_s = k_{-1}/k_1$, $K_1 = k_{-2}/k_2$ and $K = k_{-1}' + k_{-2}' + k_4$.

This predicts, therefore, a linear double-reciprocal plot. The plot of $1/k_{\text{obs}}$ versus $[I]$ or the slope of the double-reciprocal plot (Figure 2 of the main paper) versus $[I]$ and the y -intercept of the double-reciprocal plot versus $[I]$ should be hyperbolic.

(c) Quasi-equilibrium conditions

This situation arises when the product release steps in Scheme 2 of the main paper are much slower compared with other steps in the network.

The observed rate is given by eqn. (A4)

$$k_{\text{obs}} = \frac{k_3(1 + k_4[I]/k_3K_1') / (1 + [I]/K_1')}{1 + K_s/[S] \{ (1 + [I]/K_1') / (1 + [I]/K_1') \}} \quad (\text{A4})$$

where $K_1 = k_{-2}/k_2$, $K_1' = k_{-2}'/k_2'$, are the inhibition constants.

Therefore plots of $1/k_{\text{obs}}$ versus $1/[S]$ will be straight lines for fixed values of $[I]$. The plot of both slope versus $[I]$ as well as the y -intercept versus $[I]$ of these lines will be hyperbolic.