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Effects of pH and temperature on the reaction of milk xanthine oxidase with 1-methylxanthine

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The reaction of milk Xanthine Oxidase (XO) with 1-methylxanthine has been investigated by steady state and stopped flow transient kinetic studies to understand the effect of a methyl group substituent on the purine ring. The pH dependence of the steady state kinetic parameter (V_{max}/K_m) shows a bell-shaped curve implying at least two ionisable groups are involved in the binding of XO with 1-methylxanthine, the higher pK_a 7.7 corresponding to ionisation of the substrate and the lower one 6.2 to the enzyme (possibly Glu-1261 by analogy to Glu-869 of aldehyde oxidoreductase from *Desulfovibrio gigas*). The temperature dependence of the steady state and transient kinetic studies suggests the existence of at least one molecular intermediate during breakdown of the enzyme–substrate complex. The thermodynamic parameters of the microscopic rate constants were determined from the temperature dependence studies.

Introduction

Milk Xanthine Oxidase (Xanthine: Oxygen Oxidoreductase, E.C. 1.2.3.2.) is a complex metallo-flavo enzyme which catalyses the oxidation of xanthine in the presence of oxygen to form uric acid. The enzyme has two independent subunits each containing four redox centers: a molybdenum(VI) center, two iron–sulfur (2Fe/2S) clusters and a flavin adenine dinucleotide (FAD) unit.[1–3] The catalytic cycle of Xanthine Oxidase (XO) consists of two half reactions: reductive and oxidative. The reductive half reaction (*i.e.* conversion of xanthine into uric acid) takes place at the molybdenum center of the enzyme,[4,5] while the oxidative half reaction (*i.e.* conversion of oxygen into peroxide and superoxide) takes place at the FAD center.[6] In the process of the turnover, an intramolecular electron transfer (ET) takes place within the four redox centers of the enzyme. Steady state and transient kinetic studies of the reaction of XO with its physiological substrate xanthine and other substrates have been reported,[7–13] and the rates of reaction have been observed to vary with substrate. Olson *et al.* have investigated the steady state and transient kinetics of the reaction of xanthine with XO and suggested a two-step breakdown of the enzyme–substrate complex.[7] More recently, Mondal and Mitra[12] have reported the temperature dependence of this reaction and have suggested the formation of at least two molecular intermediates. The reaction of 1,7-dimethylxanthine with XO has been found to be very slow compared to that of xanthine[14] and no intermediate was reported during the breakdown of the enzyme-substrate complex. It is found that the reaction of 1-methylxanthine with XO is similar to that of xanthine but its rate is slower. To determine the effect of substitution of a methyl group on the purine ring we have carried out pH and temperature dependent studies of the reaction of XO with 1-methylxanthine by steady state and transient kinetics.

Materials and methods

Xanthine oxidase was isolated and purified from fresh unpasteurized cow's milk by the reported procedure.[12,15,16] The activity of the enzyme was measured spectrophotometrically by monitoring the formation of uric acid from xanthine at 295 nm. The calculated AFR (activity to flavin ratio) value of the enzyme was in the range 90–110 which corresponds to 50–60% functional enzyme.[17] The concentration of the enzyme was determined spectrophotometrically by using its molar absorption coefficient of 37,800 M^{-1} cm^{-1} at 450 nm.[15]

The EDTA used for the isolation of XO was removed by dialysing it thoroughly against the buffer. In all experiments Millipore milli-Q quality water was used. The experiments were done in 100 mM Tris buffer at pH > 7.6 and in 100 mM NaH_2PO_4 buffer at pH < 7.6.

All spectrophotometric experiments were performed on a Shimadzu UV-2100 spectrophotometer. A pH meter from Orion Research was used to measure the pH of the sample. A temperature controller connected to the UV-visible spectrophotometer maintained the temperature of the solution inside the cuvette for the steady state kinetic experiments. The stopped flow kinetic experiments were performed on a HITECH SF-61 stop flow machine with four microprocesser-controlled syringes. The transient reductive half reaction was studied by loading the enzyme and 1-methylxanthine in two separate syringes and made anaerobic by prolonged purging with oxygen free argon gas. The two separate solutions were mixed in a mixing chamber and the reaction was maintained under anaerobic conditions. The temperature of the syringe reservoir was maintained within ±0.5 °C using a water circulating bath. The measurements were done spectrophotometrically by monitoring the wavelength of interest. Kinetic data for the rapid kinetics were analysed by fitting using an exponential function and HITECH software.

Analysis of kinetic data

Steady state kinetics. The reductive half reaction of XO with its substrate can be described by considering that the substrate binds to XO very rapidly and forms an enzyme–substrate complex which subsequently breaks down to form product,[18] eqn. (1) where S is substrate. The steady state rate equation (2)

$$(XO)_{ox} + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (XO)_{ox}\cdot S \overset{k_2}{\rightleftharpoons} \text{product} + (XO)_{red} \quad (1)$$

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$$\frac{1}{\gamma_0} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \times \frac{1}{[S]_0} \quad (2)$$

can be derived from the above reaction scheme under the condition of $[O_2]_0 \gg K_m^{O_2}$,[19,20] where γ_0 is the initial rate of the reaction, $[S]_0$ the initial concentration of the substrate, K_m and $K_m^{O_2}$ are the apparent Michaelis constants for the substrate and oxygen respectively and V_{max} is the maximum velocity for the steady state conversion of the substrate into product. A plot of γ_0^{-1} *vs.* $[S]_0^{-1}$ will give a straight line which allows us to determine values of K_m and V_{max}.

Transient kinetics. The reductive half reaction of XO, involves transfer of electrons from the substrate to the enzyme in three consecutive steps. This results in a monophasic decrease in the enzyme absorbance at 450 nm. In the presence of an excess of the substrate compared to the enzyme, rate eqn. (3) can be derived for k_{obs}, where $K_d = k_{-1}/k_1$, k_{obs} is

$$\frac{1}{k_{obs}} = \frac{K_d}{k_2} \times \frac{1}{[S]} + \frac{1}{k_2} \quad (3)$$

the pseudo first order rate constant, K_d the dissociation constant of the enzyme–substrate complex and k_2 is the rate constant for formation of the product. Olson *et al.*[7] have shown a reaction scheme for the conversion of the enzyme–substrate complex into the product for xanthine as a substrate which can be written as in eqn. (4) where k_2' and k_2'' are the microscopic

$$(XO)_{ox}\cdot Xan \xrightarrow{k_2''} (XO)_{red}\cdot Uric\ acid \xrightarrow{k_2''} (XO)_{red} + Uric\ acid \quad (4)$$

rate constants for conversion of the enzyme–substrate complex into product.

By comparing the rate constant k_2 of eqn. (1) with k_2' and k_2'' of (4), the following relation (5) for k_2 can be derived.[18] The rate

$$\frac{1}{k_2} = \frac{1}{k_2'} + \frac{1}{k_2''} \quad (5)$$

constants can be expressed in terms of the Arrhenius equation ($k = A\exp(-E/RT)$) as in eqn. (6) where A' and A'' and E' and

$$\frac{1}{k_2} = \frac{1}{A'}\exp(E'/RT) + \frac{1}{A''}\exp(E''/RT) \quad (6)$$

E'' are the corresponding frequency factors and activation energies respectively for the two steps, T the absolute temperature and R the molar gas constant. The expression for k_2 shows a non-linear behavior with temperature. Thus, the temperature dependence of k_2 can be fitted to determine the values of A', A'', E' and E''.

Determination of thermodynamic parameters. The thermodynamic parameters associated with binding of substrate to XO were determined from the van't Hoff relation, see eqns. (7) and (8), where ΔH, ΔS and ΔG are the change in enthalpy, entropy

$$\log K_d = \left(\frac{-\Delta H}{R \times 2.303}\right) \times \frac{1}{T} + \frac{\Delta S}{R \times 2.303} \quad (7)$$

$$\Delta G = \Delta H - T\Delta S \quad (8)$$

and Gibbs free energy for formation of the enzyme–substrate complex.

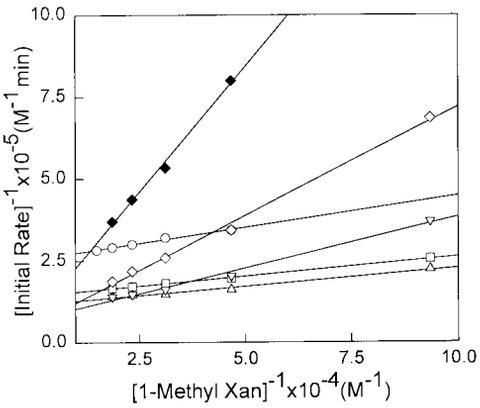

Fig. 1 Lineweaver–Burk plot for XO catalysed steady state formation of 1-methyluric acid from 1-methylxanthine using UV-visible spectrophotometry. The formation of 1-methyluric acid was measured at 295 nm. The experiments were performed at various pH (5.8–10.5). The concentration of the enzyme was 0.0249 μM. The solid lines drawn through the experimental data points at pH 5.8(○), 6.7(□), 7.6(△), 7.8(▽), 8.5(◇) and 9.1(◆). The values of K_m and V_{max} were determined from the slope and intercept of eqn. (2).

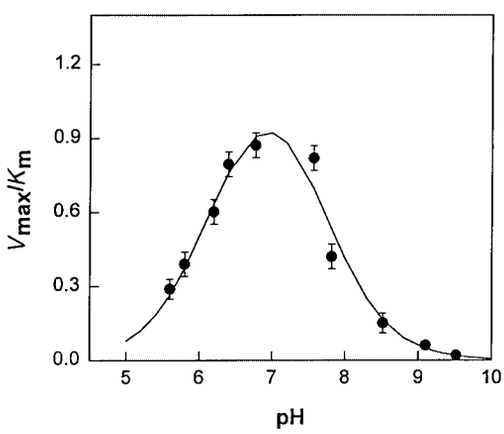

Fig. 2 Plot of V_{max}/K_m *vs.* pH for the reaction of XO with 1-methylxanthine. The solid line drawn through the experimental data is the theoretical fit to eqn. (9). The fitted pK_1 and pK_2 were 6.2 and 7.7 respectively.

Results

(a) pH dependence

The steady state XO catalysed oxidation of 1-methylxanthine to its corresponding uric acid was monitored at 295 nm at different concentrations of 1-methylxanthine. The oxygen concentration of the sample was kept constant at 515 μM. The Michaelis constant ($K_m^{O_2}$) for the binding of oxygen to XO is 50 μM.[15,21] The oxygen concentration was thus about 10 times higher than $K_m^{O_2}$.[12] The steady state kinetic experiments were carried out in the pH range 5.8–9.5. It is to be emphasized that XO is stable in this pH range. Fig. 1 shows the Lineweaver–Burk (LB) plot at various pH from which K_m and V_{max} were determined. Typical values were found to be 8.3 μM and 7.2 μM min^{-1} respectively at pH 6.8. The plot of the second order rate constant V_{max}/K_m *vs.* pH exhibits a bell shaped variation with a maximum at pH 6.8 (Fig. 2). A similar pH dependence was reported for xanthine.[13] It suggests that at least two ionisable groups are involved in the reaction of XO with 1-methylxanthine, which can be explained by considering double ionisation of the enzyme, $H_2E \overset{K_2}{\rightleftharpoons} HE \overset{K_2}{\rightleftharpoons} E$ where K_1 and K_2 are the acid equilibrium constants. Assuming that the singly protonated form of the enzyme is catalytically active, the expression (9) for V_{max}/K_m can be derived.[13] A fit of the experimental data of Fig. 2 by eqn. (9) gave pK_1 = 6.2 and pK_2 = 7.7.

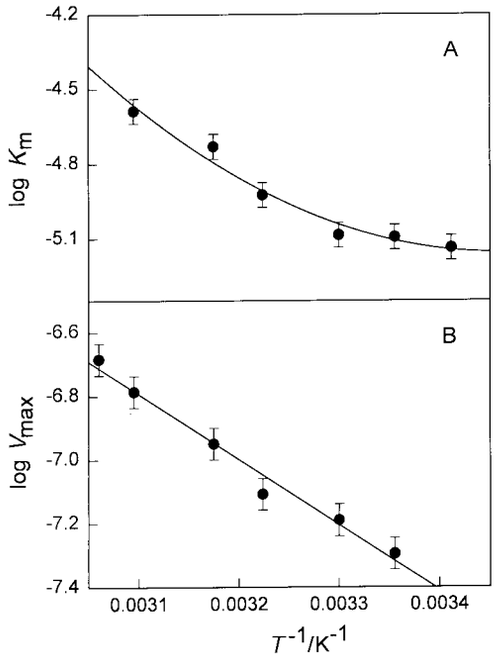

Fig. 3 Logarithmic plots of K_m (A) and V_{max} (B) *vs.* $1/T$ for the steady state conversion of 1-methylxanthine into the corresponding uric acid at pH 6.8.

$$(V_{max}/K_m) = \frac{(V_{max}/K_m)_{max}}{1 + [H^+]K_1^{-1} + K_2[H]^{-1}} \qquad (9)$$

This indicates that an ionisable group having pK_a of 6.2 must be deprotonated and that one having a pK_a of 7.7 must be protonated for the reaction to take place. The former pK_a may correspond to a functional group in the active site of the enzyme and the latter to the substrate.[13]

(b) Temperature dependence

The temperature dependence of the steady state kinetics of the reaction of 1-methylxanthine with XO was studied between 14 and 50 °C. The data were analysed as described above for pH dependent measurements, and LB plots were constructed (not shown here) at each temperature to obtain V_{max} and K_m. The logarithmic plots of V_{max} and K_m *vs.* temperature show a linear trend for V_{max} and a non-linear trend for K_m (Fig. 3). Theoretically, both V_{max} and K_m consist of several microscopic rate constants.[12] Therefore, their logarithmic plots with temperature might be expected to follow a non-linear trend. In practice, however, both linear and non-linear trends are possible depending on the relative magnitudes of the individual microscopic rate constants in V_{max} and K_m.[12] The temperature dependence of the logarithmic plots of V_{max} and K_m for xanthine was found to be similar to that observed for 1-methylxanthine. The present observation of a linear trend for log V_{max} *vs.* $1/T$ suggests that overall composite rate constants control the turnover rate of the reaction of XO with 1-methylxanthine. From the linear trend in Fig. 3B, the activation energy related to V_{max} was determined to be 9.3 kcal mol^{-1}. The non-linear behaviour of log K_m *vs.* $1/T$ (Fig. 3A) is consistent with the theoretical prediction of K_m.[12] A similar non-linear dependence of log K_m with temperature has been observed for other enzyme–substrate reactions.[22]

The transient kinetics of the reaction of XO with 1-methylxanthine were measured in the temperature range 14–50 °C. Fig. 4 shows a typical stopped flow trace of the reduction of the enzyme at 450 nm under pseudo first order conditions. The experimental data could best be fitted by a single exponential function to obtain k_{obs}. A typical value of $k_{obs} = 4.9$ s^{-1} was

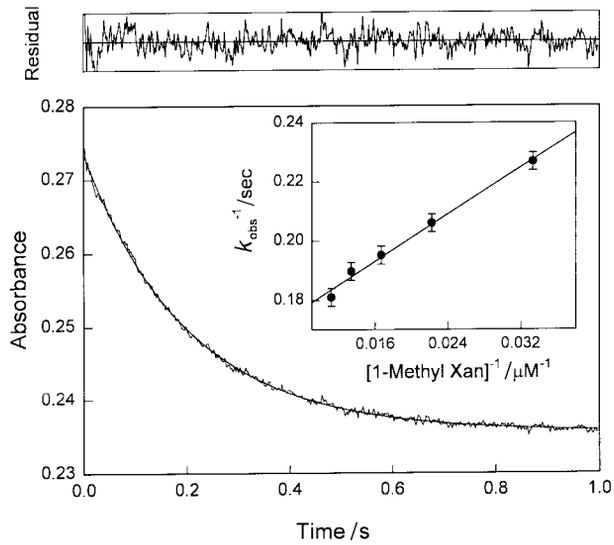

Fig. 4 Typical stopped flow trace for the anaerobic reduction of XO (5 μM) with 1-methylxanthine (75 μM) at 450 nm. The solid line drawn through the experimental trace is the computer fit to the single exponential function. $A_t = A_0 \exp(-k_{obs}t) + A_\infty$, where A_t is the absorbance at time t, A_0 the amplitude of the absorbance change, k_{obs} the observed rate constant, t the time in seconds and A_∞ the equilibrium signal absorbance (offset). The inset in the figure shows a plot of k_{obs}^{-1} *vs.* [1-Methyl Xan]$^{-1}$. The solid line drawn through the experimental data is the fit by eqn. (3).

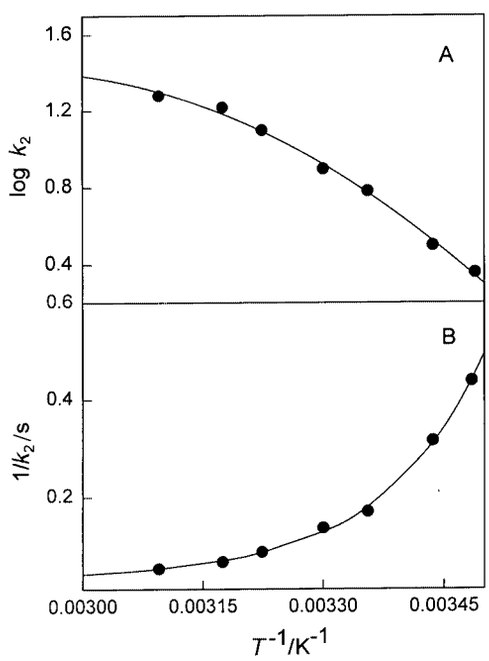

Fig. 5 (A) Logarithmic plot of k_2 *vs.* $1/T$ shows the non-linear behavior for the formation of product at pH 6.8. The solid line drawn through the experimental data points shows the trend of the plot. (B) Plot of $1/k_2$ *vs.* $1/T$ for the same experiment. The solid line drawn through the experimental data is the fit by eqn. (6). The fitted values are E_2' 1.6 kcal mol^{-1}, E_2'' 16.4 kcal mol^{-1}, A_2' 3.3×10^2 s^{-1} and A_2'' 7.6×10^{12} s^{-1}.

obtained when 75 μM 1-methylxanthine reacted with 5 μM XO. A plot of k_{obs}^{-1} *vs.* [1-Methyl Xan]$^{-1}$ gives a straight line which suggests reversible binding of 1-methylxanthine to XO with 1 : 1 stoichiometry. Using eqn. (3), typical values of k_2 and K_d were deduced: 6.0 s^{-1} and 9.0 μM at 25 °C. Using the values of K_d at various temperatures the thermodynamic parameters (ΔG, ΔH and ΔS) associated with binding of 1-methylxanthine to XO (XO + 1-methylxanthine $\rightleftharpoons$ bound complex) were determined from the linear plot of log K_d *vs.* $1/T$ (figure not shown).

Using eqns. (7) and (8), we obtain $\Delta G = 6.9$ kcal mol^{-1}, $\Delta H = 10$ kcal mol^{-1} and $\Delta S = 10.8$ cal K^{-1} mol^{-1}. The Arrhenius plot of k_2 shows a non-linear trend (Fig. 5A). The plot of $1/k_2$ *vs.* $1/T$ also shows a non-linear trend (Fig. 5B). Eqn. (6) was used to fit the experimental data of Fig. 5B (see the solid line). Eqn. (6) has four adjustable parameters, and hence their individual values cannot uniquely be determined by fitting the experimental data. However, an estimate of their values was made by the least squares fit which gave: $E_2' = 1.6$ kcal mol^{-1}, $E_2'' = 16.4$ kcal mol^{-1}, $A_2' = 3.3 \times 10^2$ s^{-1} and $A_2'' = 7.6 \times 10^{12}$ s^{-1}.

Discussion

The K_m and V_{max} values for 1-methylxanthine were found to be 8.3 μM and 7.2 μM min^{-1} at 25 °C and pH 6.8. The corresponding values for xanthine were reported as 4.0 μM and 15 μM min^{-1} at 25 °C and pH 6.8.[18] This indicates that the binding affinity of 1-methylxanthine to XO is lower than that of xanthine. It also suggests that the rate of the reaction of XO with 1-methylxanthine is slower than with xanthine. These may be due to the presence of a methyl group at N-1 in 1-methylxanthine which sterically hinders the binding of 1-methylxanthine to XO. V_{max}/K_m exhibits a bell shaped pH dependence as expected from the double ionisation of the enzyme, considering the singly protonated form of the enzyme to be catalytically active. Eqn. (9) gave a good fit to the experimental data of Fig. 2 and the pK_as were found to be 6.2 and 7.7. The pH dependence of V_{max}/K_m represents ionisation of the free enzyme and free substrate. It is to be noted that, with regard to the high pH limb of the curve, there is no known ionisation of the enzyme in this pH range.[13] Therefore, the pK_a 7.7 corresponds to ionisation of the substrate. In the case of xanthine V_{max}/K_m also exhibits a bell shaped pH dependence.[13] It is known that xanthine has a pK_a of 7.3 which has been attributed to ionisation of its N-1 proton.[23] It is suggested that the neutral form of the substrate is required for binding to the enzyme.[13] The presence of the methyl group at N-1 in 1-methylxanthine replaced the ionisation of the hydrogen at N-1. Therefore, the pK_a 7.7 of 1-methylxanthine arises from ionisation of its N-9 proton. The pK_a of 1-methylxanthine obtained from our studies is not very much different from that of xanthine. This suggests that the presence of the methyl group does not seem to have much effect on the ionisation of the substrate. The implication is that the protonated (neutral) form of the substrate is required for binding to the enzyme. This is consistent with the abstraction of the proton from C-8, as the negative charge of the ionised substrate destabilises the accumulating negative charge on C-8 for the deprotonation. The pK_a 6.2 must be attributed to the ionisation of a residue in the active site of the enzyme. Recently, the crystal structure of aldehyde oxidoreductase from *Desulfovibrio gigas* was solved at 1.8 Å, and on the basis of this structure it was proposed that Glu-869 at the active site abstracts a proton from molybdenum bound water/hydroxide and thereby facilitates nucleophilic attack on the substrate.[24] Xanthine oxidase has good amino acid sequence homology with aldehyde oxidoreductase in the vicinity of their molybdenum center that includes this glutamate residue (Glu-869 of the *D. gigas versus* Glu-1261 of the bovine enzyme). We suggest that Glu-1261 of XO plays a role analogous to that proposed for Glu-869 of aldehyde oxidoreductase. Although the observed pK_a 6.2 is rather high for a glutamate it is not surprising. The pK_a of Glu-35 in lysozyme is reported to be 6.5.[25]

K_m and V_{max} for the reaction of xanthine with XO are known to be combinations of several microscopic rate constants.[12] The linear dependence of log V_{max} with $1/T$ for the reaction of 1-methylxanthine with XO suggests that V_{max} is controlled by one rate constant (either k_2 or k_4).[12] Since the Arrhenius plot of k_2 shows non-linear behavior (see Fig. 5A), the linear dependence of log V_{max} with $1/T$ must arise from the contribution of k_4. This is consistent with the observation of Olson *et al.*[21] that k_4 is higher than k_2 in the case of xanthine as a substrate. K_m is expressed as: $V_{max}(k_2 + k_{-1})/E_0k_1k_2$.[12] Since V_{max} shows a linear Arrhenius plot with negative slope, the non-linear behavior of K_m must be attributed to the factor $(k_2 + k_{-1})/k_1k_2$. The Arrhenius plot of k_2 shows non-linear behavior, so the non-linearity of log K_m with $1/T$ arises from the contribution of k_2. The ratio V_{max}/K_m determines the specificity of the enzyme towards the substrate and it was found that this ratio is independent of temperature for 1-methylxanthine. However, for xanthine it has been observed that it increases with increasing temperature.[12]

We have determined K_d due to the binding of 1-methylxanthine to XO from the transient kinetic measurements. The thermodynamic parameters associated with the binding at various temperatures were determined from the temperature dependence of K_d. The positive value of ΔG indicates that the dissociation of the enzyme–substrate complex is energetically unfavorable. The positive values of ΔH and ΔS indicate that the binding of XO to 1-methylxanthine is an endothermic process and the dissociation of the enzyme–substrate complex is entropy-driven.

Olson *et al.*[7,21] and Hille and Massey[18] suggested the involvement of one intermediate in the breakdown of the enzyme–substrate complex (XO_{ox}·Xan → XO_{red} + Urate) in the reaction of XO with xanthine. Kim *et al.*[13] also studied the reaction of XO with xanthine and suggested the existence of multiple intermediates during the breakdown of the enzyme-substrate complex. The temperature dependent transient kinetics of the reaction of XO with xanthine has also been investigated by Mondal and Mitra.[12] Their investigations indicate non-linear plots of log k_2 (and $1/k_2$) *vs.* $1/T$. These results suggest that at least two intermediates are involved during the breakdown of the XO_{ox}·Xan complex to the product (uric acid).[12] In the present measurements, similar attempts have been made to obtain further insights into the breakdown of the XO_{ox}·1-Methyl Xan complex to the product (1-methyluric acid). Eqn. (6) was used to fit the experimental data of Fig. 5B. Eqn. (4) considers the existence of only one intermediate for the breakdown of XO_{ox}·1-Methyl Xan to the product. Further, a critical inspection of eqn. (6) and the experimental data in Fig. 5B reveals that both the activation energies (E_2' and E_2'') can not be negative. It is however possible that both the activation energies are positive or with opposite signs. The results from our analysis show that eqn. (6) fits very well the experimental data of Fig. 5B (see the solid line) only when both the activation energies are considered to be positive (1.6 and 16.4 kcal mol^{-1}). It therefore appears that the breakdown of the XO·1-Methyl Xan complex to the product involves the existence of at least one intermediate.

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