DALTON
FULL PAPER

Reaction of milk xanthine oxidase with *o*-phenanthroline and 1,7-dimethylxanthine: a mechanistic study

Apurba Kumar Sau, Madhu Sudan Mondal and Samaresh Mitra *

Department of Chemical Sciences, Tata Institute of Fundamental Research, Homi Bhabha Road, Colaba, Mumbai 400 005, India. E-mail: smitra@tifr.res.in

Received 8th September 1998, Accepted 12th October 1998

The kinetics of the reaction of milk xanthine oxidase (XO) with *o*-phenanthroline and 1,7-dimethylxanthine are investigated by optical and circular dichroism (CD) spectroscopic techniques. *o*-Phenanthroline and 1,7-dimethylxanthine are observed to react with XO, resulting in a slow decrease in the absorbance and CD signals of the enzyme. The results suggest the existence of a multi-binding mechanism in the reaction of XO with *o*-phenanthroline with two molecules of the substrate binding to one molecule of the enzyme. However, 1,7-dimethylxanthine binds reversibly with the enzyme with 1 : 1 stoichiometry. The kinetic effect experienced individually by the flavin adenine dinucleotide (FAD) chromophore of XO has been separately resolved, and shows that at least one molecule of *o*-phenanthroline may bind near the FAD centre of the enzyme. 1,7-Dimethylxanthine is expected to bind to the molybdenum centre in analogy with xanthine.

Introduction

Xanthine oxidase (xanthine: oxygen oxidoreductase, EC 1.2.3.2; XO) is a complex metalloflavo enzyme with two independent subunits each containing four redox centres; a molybdenum(VI) centre, two iron–sulfur (2Fe-2S) clusters, and a flavin adenine dinucleotide (FAD) unit.[1–3] It catalyses the oxidation of xanthine in the presence of oxygen to form uric acid, and in the process, hydrogen peroxide or superoxide radical is formed from oxygen. The complete catalytic reaction of XO consists of two half reactions. During the first half of the catalytic reaction, called reductive half reaction (RHR), xanthine binds at the molybdenum centre and transfers two electrons to the enzyme.[4] In the process the enzyme is reduced and uric acid is formed from xanthine as the oxidation product. During the second half of the catalytic reaction, called oxidative half reaction (OHR), molecular oxygen oxidises the reduced form of XO, and peroxide and/or superoxide radical is formed from oxygen. The RHR takes place at the molybdenum centre of the enzyme while OHR takes place at the FAD.[5]

Once electrons are transferred from xanthine to XO, there is a rapid intramolecular electron-equilibration process among the four redox centres of the enzyme.[6] The rates of these electron-equilibration processes are determined by the relative magnitude of the mid point reduction potentials of the four redox centres.[7] It is known that the potentials of FAD and Fe/S chromophores are different.[8] Therefore, the rates at which the FAD and Fe/S centres are reduced would be different. In other words, the kinetics of reduction of FAD and Fe/S centres would be uneven. Among the redox centres of XO, Fe/S centres and FAD have characteristic circular dichroism (CD) and absorption bands. Upon transfer of electrons to XO, the intensities of these bands are reduced. Hence, the kinetics of the reduction of FAD and Fe/S centres can be monitored by measuring the decrease in absorbance and CD signals of the enzyme with time. Several fast kinetic studies on the reduction of xanthine oxidase chromophores by xanthine have earlier been reported.[7] The reaction of xanthine with XO is quite fast, which makes it difficult to obtain mechanistic information on the binding of the substrate to the enzyme. Further, in many biological reactions, the substrate binding processes involve multiple association of the substrate molecules with the enzyme but these binding processes are generally too fast to be experimentally detected. We report here the results of the reaction of two non-physiological substrates, *o*-phenanthroline and 1,7-dimethylxanthine (3,7-dihydro-1,7-dimethyl-1*H*-purine-2,6-dione) with XO since their reaction with this enzyme is much slower than xanthine (the rate of reduction of XO, for example, with *o*-phenanthroline is approximately 10^{-4} times less than with xanthine),[9] and is therefore convenient for kinetic resolution of the substrate binding process. The reaction of *o*-phenanthroline with proteins is known to be important, as it is involved in the control of aminoacylase function.[10,11] It has been shown that *o*-phenanthroline induces the soxRS regulon anaerobically by stimulation of soxS transcription.[11] 1,7-Dimethylxanthine is a substrate analogue of the physiological substrate xanthine.

In the present paper, the reduction rate of FAD and Fe/S centres of XO by *o*-phenanthroline and 1,7-dimethylxanthine has been utilised to understand the substrate binding mechanism. The results indicate involvement of a multi-binding mechanism for the reaction of *o*-phenanthroline with XO, while reversible binding of one molecule of 1,7-dimethylxanthine to one molecule of the enzyme, is observed.

Experimental

Xanthine oxidase was isolated and purified from fresh unpasteurized cow's milk by the reported procedure.[12–14] 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) was in the range which corresponds to 50–60% functional form of the enzyme.[15] The PFR (protein to flavin ratio) of the enzyme was found to be 5.4. The concentration of the enzyme was determined spectrophotometrically by using the molar absorption coefficient as 37,800 M^{-1} cm^{-1} at 450 nm.[12]

The experiments were done in 100 mM analytical NaH_2PO_4 buffer, pH 5.8. *o*-Phenanthroline and 1,7-dimethylxanthine were of analytical grade. The H_4EDTA used for the isolation of XO was removed thoroughly by dialysing against the buffer. In all the experiments millipore milli-Q quality water was used.

Spectrophotometric experiments were performed on a Shimadzu UV-2100 spectrophotometer. CD spectra were recorded on a J-600 (Jasco) spectropolarimeter. The anaerobic experiments were carried out in an anerobic cuvette that was fitted with rubber septa. All spectrophotometric and CD

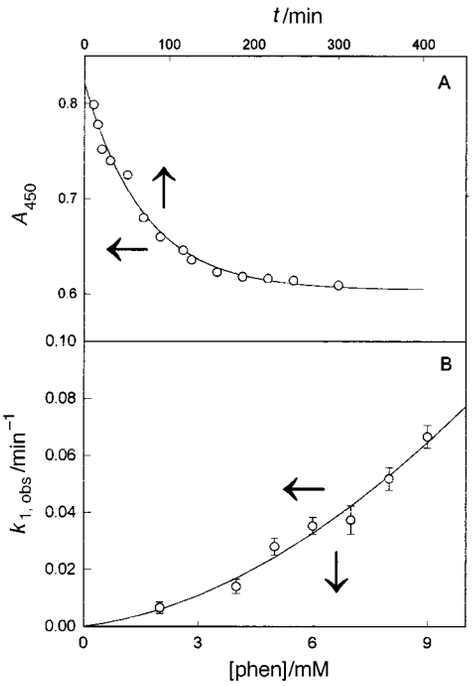


Fig. 1 (A) The change in absorbance of XO (18.6 μM) with time at 450 nm due to the anaerobic reduction with *o*-phenanthroline (4 mM) at pH 5.8. The solid line drawn through the experimental points is the computer fit to the single exponential function: $A_t = A_0 \exp(-k_{1,obs}t) + B$, where A_t is the change in absorbance with time, $k_{1,obs}$ = observed rate constant, t is the time in min and B is the equilibrium absorbance signal. The $k_{1,obs}$ was found to be 0.014 min^{-1}. (B) Plot of $k_{1,obs}$ *vs.* [*o*-phenanthroline] obtained from the kinetic trace(s) at various concentrations of *o*-phenanthroline. The solid line drawn through the experimental points shows the theoretical fit to eqn. (1).

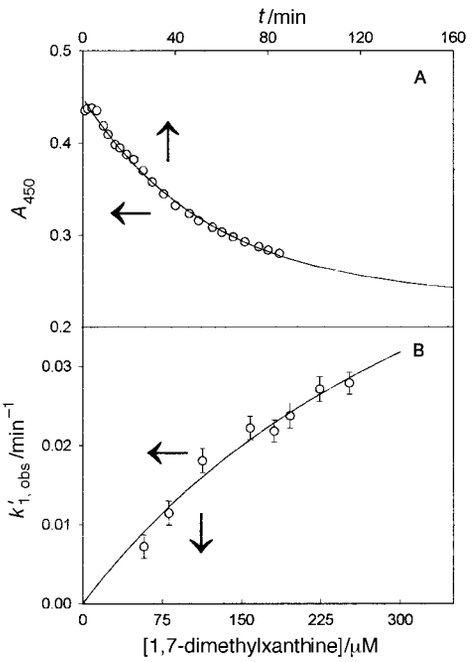


Fig. 2 (A) The change in absorbance of XO (10.5 μM) with time at 450 nm due to the anaerobic reduction with 1,7-dimethylxanthine (112 μM) at pH 5.8. The solid line drawn through the experimental points is the computer fit to the single exponential function: $A_t = A_0 \exp(-k'_{1,obs}t) + B$. The $k'_{1,obs}$ was found to be 0.018 min^{-1}. (B) Plot of $k'_{1,obs}$ *vs.* [1,7-dimethylxanthine] obtained from the kinetic trace(s) at various concentrations of 1,7-dimethylxanthine. The solid line drawn through the experimental points shows the theoretical fit to eqn. (2).

experiments were performed at 26 ± 0.5 °C with prolonged purging of the cuvette by high-quality argon gas. The argon gas was passed above the surface of the anaerobic sample during the experiments to ensure a completely anaerobic atmosphere.

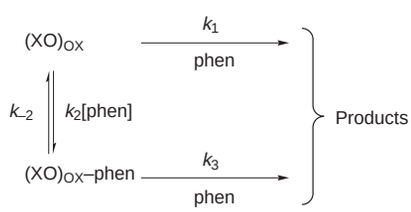
Results

The FAD and Fe/S centres in XO show an absorbance at 450 nm. The decrease in the absorbance at this wavelength in the presence of *o*-phenanthroline or 1,7-dimethylxanthine is due to the reduction of both FAD and Fe/S centres.

Optical spectral study

Fig. 1A and 2A show decreases in the absorbance at 450 nm with time upon anaerobic reduction of XO by *o*-phenanthroline and 1,7-dimethylxanthine. The decrease in the absorbance could be fitted to a single exponential function to obtain an overall observed rate constant (k_{obs}). The typical values of the observed rate constants for the reaction of these two substrates are: $k_{1,obs} = 0.014$ min^{-1} (*o*-phenanthroline, concentration 4 mM); $k'_{1,obs} = 0.018$ min^{-1} (1,7-dimethylxanthine; concentration 112 μM).

We investigated the anaerobic reduction of XO in the 2–9 mM concentration range of *o*-phenanthroline. The kinetic traces fit well to single exponential function, yielding the values of $k_{1,obs}$ at various concentrations of phenanthroline. Fig. 1B shows that $k_{1,obs}$ increases with the increase in the concentration of *o*-phenanthroline, but the trend of its variation is convex with respect to abscissa (*i.e.* substrate concentration). This type of variation of $k_{1,obs}$ with substrate concentration has been observed for the reaction of horseradish peroxidase and lactoperoxidase with *o*-phenanthroline and 1,7-dimethylxanthine,[16,17] and was interpreted considering a reaction scheme that includes multiple binding of substrates with the enzyme.[16,17] Therefore, considering a similar multiple substrate binding mechanism for the reaction of *o*-phenanthroline with XO, Scheme 1 may be suggested, where $(XO)_{ox}$-phen is a



Scheme 1 Multi-binding mechanism.

complex formed between the oxidised form of XO and *o*-phenanthroline; k_1 and k_3 are the apparent second order rate constants for the formation of products; k_2 and k_{-2} are the forward and backward rate constants for the formation of XO-phen complex from XO and phenanthroline. Eqn. (1) for $k_{1,obs}$ can be derived from Scheme 1.

$$k_{1,obs} = \frac{k_1[S] + \{k_2k_3[S]^2 \times (k_3[S] + k_{-2})^{-1}\}}{1 + \{k_2[S] \times (k_3[S] + k_{-2})^{-1}\}} \qquad (1)$$

The experimental data in Fig. 1B fit well to eqn. (1) (see the solid line in Fig. 1B), which supports the minimum model for the multiple binding mechanism given in Scheme 1. Eqn. (1) has four adjustable parameters, and hence their individual values cannot be uniquely determined by fitting to the experimental data. However, an estimate of their values was made by the least square fit which gave: $k_1 = 1.6$ M^{-1} min^{-1}, $k_2 = 123$ M^{-1} min^{-1}, $k_{-2} = 18$ min^{-1} and $k_3 = 104$ M^{-1} min^{-1}.

The observed rate constant ($k'_{1,obs}$) for the reaction of 1,7-dimethylxanthine with XO shows hyperbolic increase with the substrate concentrations (Fig. 2B). This can be explained by considering the following minimum mechanism (Scheme 2),

$$(XO)_{ox} + \text{1,7-dimethylxanthine} \underset{k'_{-1}}{\overset{k'_1}{\rightleftharpoons}} (XO)_{ox} \bullet \text{1,7-dimethylxanthine} \xrightarrow{k'_2} (XO)_{red} + \text{1,7-dimethyluric acid}$$

Scheme 2

where 1,7-dimethylxanthine forms a reversible complex with XO leading to the formation of product.

Eqn. (2) for the observed rate constant ($k'_{1,obs}$) can be derived for the above mechanism, where, S is 1,7-dimethylxanthine, $k'_D = k'_{-1}(k'_1)^{-1}$ and k'_2 is the rate constant for the formation of product from the complex. The solid line in Fig. 2B is a fit to eqn. (2). The values of k'_2 and k'_D obtained from

$$k'_{1,obs} = \frac{k'_2[S]}{K'_D + [S]} \qquad (2)$$

such a fit are 0.08 min^{-1} and 440 μM respectively. The results indicate a weak binding affinity of 1,7-dimethylxanthine with XO.

Circular dichroism study

Xanthine oxidase shows a rather intense CD spectrum, which strongly resembles that of spinach ferredoxin and other 2Fe/2S proteins.[18] The intensity of the CD spectrum in XO is nearly twice that of the Fe/S proteins, and is believed to arise from the two iron–sulfur centres of XO,[19] the flavin and molybdenum centres making little or no contribution to the CD spectrum. Thus circular dichroism spectroscopy is very convenient to investigate the reduction of the iron–sulfur centres in XO. Our CD spectrum of XO matched well with the previously reported one.[19] The spectra were recorded as a function of time in the presence of phenanthroline (data not shown), and time-profiles of these spectra were obtained at 450 nm. Fig. 3A shows a typical plot of the variation of CD intensity with time in the presence of phenanthroline. The data in Fig. 3A were fitted to a single exponential function to obtain the overall observed rate constant $k_{2,obs}$. A typical value of $k_{2,obs}$ was 0.01 min^{-1} at 4 mM concentration of *o*-phenanthroline. Fig. 3B shows the variation of $k_{2,obs}$ with the concentration of *o*-phenanthroline, which is very similar to that observed in the optical spectral studies (see Fig. 1B). The solid line in Fig. 3B is the theoretical fit to an equation similar to eqn. (1). The results obtained from the CD and absorbance measurements are thus in qualitative agreement and suggest that a multi-binding mechanism is operative for the reaction of *o*-phenanthroline with XO.

The CD studies of XO with 1,7-dimethylxanthine showed that the data fitted well to a single exponential function, which gave the observed rate constants, $k'_{2,obs} = 0.017$ min^{-1} at 115 μM concentration of 1,7-dimethylxanthine. The variation of the observed rate constants with the concentration of 1,7-dimethylxanthine obtained from the CD measurements was found to be similar to that observed from the absorbance measurements. Thus the CD and optical spectral measurements both suggest the formation of a reversible complex for the binding of 1,7-dimethylxanthine with XO.

Resolution of the kinetic effects of substrates at the FAD centre of XO

The FAD and Fe/S centres in XO show absorbance at 450 nm. However, the Fe/S centres additionally absorb at 550 nm. Thus any decrease in the absorbance at 450 nm may be attributed to the reduction of both FAD and Fe/S centres, but the decrease in the absorbance at 550 nm can only be due to the reduction of Fe/S centres. It is therefore possible to separate out the contribution of the FAD centre to the absorbance at 450 nm from the

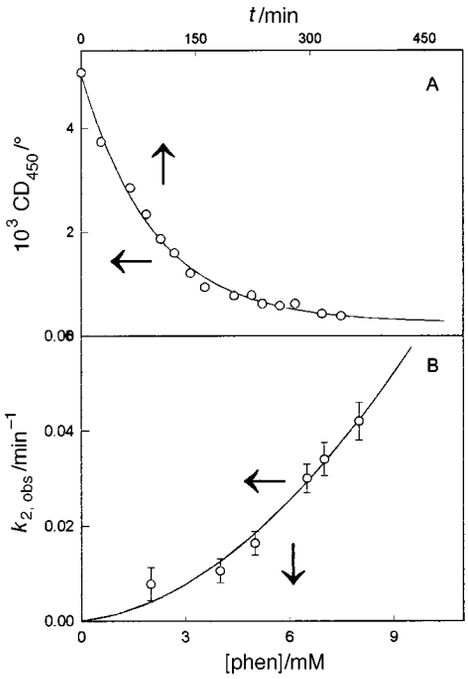


Fig. 3 (A) The variation in CD intensity in ° at 450 nm due to the anaerobic reduction of XO (18.6 μM) with *o*-phenanthroline (4 mM). The solid line drawn through the experimental data points is the computer fit to the single exponential: $y = m \exp(k_{2,obs}t) + c$, where y is the change in CD intensity with time, $k_{2,obs}$ is the observed rate constant, t is the time in min and c is the equilibrium CD signal. The $k_{2,obs}$ was found to be 0.01 min^{-1}. (B) Plot of $k_{2,obs}$ *vs.* [*o*-phenanthroline] at 450 nm from CD studies. The solid line shows the theoretical fit to eqn. (1).

known absorption coefficients of Fe/S centres at 450 and 550 nm. The absorbances at 450 and 550 nm were determined from the optical spectra of XO in the presence of *o*-phenanthroline and 1,7-dimethylxanthine. The contribution to the absorbance separated by the FAD centre can be obtained from eqn. (3),

$$\text{Absorbance due to FAD} = A_{450} - \frac{\varepsilon^{Fe/S}_{450}}{\varepsilon^{Fe/S}_{550}} \times A_{550} \qquad (3)$$

where A_{450} and A_{550} are the absorbances at 450 nm and 550 nm respectively; $\varepsilon^{Fe/S}_{450}/\varepsilon^{Fe/S}_{550}$ is the ratio of the absorption coefficients of Fe/S centres at 450 nm and 550 nm; this ratio has been found to be about 1.87 : 1 from the spectral studies of native and deflavo XO.[5]

Following this procedure, a time-profile of the changes in the absorbance of the FAD centre in the reaction of XO with *o*-phenanthroline and 1,7-dimethylxanthine was determined. Fig. 4A shows such a kinetic trace for *o*-phenanthroline which fits well to a single exponential function to give observed rate constant $k_{3,obs}$. A typical value of $k_{3,obs}$ obtained from such an exponential fit was 0.039 min^{-1} at 4 mM concentration of *o*-phenanthroline. The values of $k_{3,obs}$ obtained at different concentrations of *o*-phenanthroline are plotted in Fig. 4A (inset). The figure shows a simple hyperbolic saturation curve, which can be explained by the minimum mechanism shown in Scheme 3, where, $K''_D = \frac{k''_{-1}}{k''_1}$ and k''_2 is the rate constant for the formation of product from the complex.

$$\text{FAD} + \text{phen} \underset{k''_{-1}}{\overset{k''_1}{\rightleftharpoons}} \text{FAD} - \text{phen} \xrightarrow{k''_2} \text{Products}$$

Scheme 3

For $k_{3,obs}$ eqn. (4) can be derived from the above mechanism.

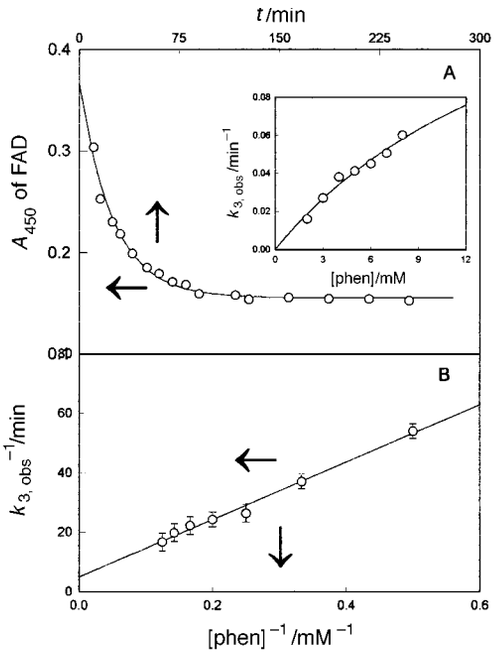


Fig. 4 (A) The plot of FAD absorbance at 450 nm due to the anaerobic reduction in the presence of *o*-phenanthroline (4 mM). The concentration of the enzyme was kept at 18.6 μM. The absorbance of only FAD centres were determined as described in the text. The solid line drawn through the experimental data points is the fit to a single exponential function: $A_t = A_0 \exp(-k_{3,obs}t) + B$. The $k_{3,obs}$ was found to be 0.039 min^{-1}. Plots of $k_{3,obs}$ *vs.* [*o*-phenanthroline] from the above trace(s) are shown as an inset. (B) The double reciprocal plot of the data shown in inset Fig. 4A. The solid line is the theoretical fit based on eqn. (4).

$$\frac{1}{k_{3,obs}} = \frac{K''_D}{k''_2} \times \frac{1}{[phen]} + \frac{1}{k''_2} \qquad (4)$$

The double reciprocal plot of $1/k_{3,obs}$ *vs.* 1/[phen] shows that the data fit well to eqn. (4) (see Fig. 4B). The values of k''_2 and K''_D obtained from such a fit are 0.17 min^{-1} and 16 mM respectively. These results indicate a very slow rate of reduction of the FAD centre and a weak apparent binding affinity of phenanthroline with the FAD centre.

We have used the same procedure to construct a time-profile of changes in the absorbance of FAD in the reaction of 1,7-dimethylxanthine with XO. The data can be best fitted to a single exponential function, which gave the observed rate constants. A typical value of the observed rate constant ($k'_{3,obs}$) from such an exponential fit was 0.03 min^{-1} at 196 μM of 1,7-dimethylxanthine. The variation of the observed rate constants with the increase in the concentration of 1,7-dimethylxanthine shows similar behaviour to that observed in case of *o*-phenanthroline. The values of k''_2 and K''_D obtained from this method are 0.06 min^{-1} and 228 μM respectively.

Discussion

Under anaerobic conditions, *o*-phenanthroline and 1,7-dimethylxanthine reduce XO by transferring electrons to its four redox centres. The oxidation product of 1,7-dimethylxanthine was found to be the corresponding uric acid. The oxidation product of *o*-phenanthroline could not be determined by optical spectroscopy. It is expected that XO which is a mild oxidising agent, may oxidise one of the N-positions of *o*-phenanthroline leading to the formation of phenanthroline *N*-oxide.

We have deduced three observed rate constants in the reaction of XO with these two substrates: $k_{1,obs}$ ($k'_{1,obs}$), $k_{2,obs}$ ($k'_{2,obs}$) and $k_{3,obs}$ ($k'_{3,obs}$); $k_{1,obs}$ (or $k'_{1,obs}$) has been obtained from the optical spectral studies, and monitors both FAD and the two Fe/S centres; $k_{2,obs}$ (or $k'_{2,obs}$) has been deduced from circular dichroism measurements monitoring only the two Fe/S centres. The values of the observed rate constants for the binding of *o*-phenanthroline with XO ($k_{1,obs}$ and $k_{2,obs}$) are found to be similar (0.014 and 0.010 min^{-1}). The values of the corresponding observed rate constants for 1,7-dimethylxanthine ($k'_{1,obs}$ or $k'_{2,obs}$) are also very similar (0.018 and 0.017 min^{-1}).

The resolved rate constant for *o*-phenanthroline ($k_{3,obs}$ = 0.039 min^{-1}) is found to be much larger than $k_{1,obs}$ and $k_{2,obs}$. The corresponding value of the resolved rate constant ($k'_{3,obs} = 0.030$ min^{-1}) for 1,7-dimethylxanthine is however smaller than the resolved rate constants ($k'_{1,obs}$). The electron transfer to the redox centres of XO is known to be determined by the reduction potential of the FAD centre. Hence the large difference between $k_{3,obs}$ and $k_{1,obs}$ (or $k_{2,obs}$) for the reaction of *o*-phenanthroline with XO suggests that the binding of *o*-phenanthroline to XO increases substantially the positive reduction potential of FAD, while in the case of 1,7-dimethylxanthine binding the reduction potential is altered to a lesser extent. It is found that the rate constant (k''_2) for the reduction of FAD in the presence of *o*-phenanthroline is higher than that for 1,7-dimethylxanthine. The differences in rate constant (k''_2) for the reduction of FAD may be due to the intrinsic differences between the two structures of the substrates. The close agreement between Scheme 1 and the variation in the observed rate constant with the concentration of *o*-phenanthroline (see Fig. 1B and 3B) suggested a multiple binding mechanism. Scheme 1 further suggests that two molecules of *o*-phenanthroline might be binding to one molecule of the enzyme. These results therefore indicate that one of the binding sites of *o*-phenanthroline may be near the FAD centre, affecting the reduction potential. The relatively small increase in the resolved rate constant for 1,7-dimethylxanthine suggests that the binding site of this substrate is perhaps further from the FAD centre. We further note that the data of Fig. 2B indicates a 1:1 binding stoichiometry for 1,7-dimethylxanthine. The physiological substrate xanthine is known to bind at the molybdenum centre of the enzyme.[5] Since 1,7-dimethylxanthine is a substrate analogue of xanthine, this substrate may also bind at the molybdenum centre of the enzyme. It is seen from Fig. 1B and 3B that at low concentrations of *o*-phenanthroline the observed rate constants ($k_{1,obs}$ and $k_{2,obs}$) increase slowly but at higher concentrations of the substrates an appreciable amount of *o*-phenanthroline bound XO is formed. It thus appears that initially one molecule of *o*-phenanthroline binds to the enzyme at a specific site and as the concentration of *o*-phenanthroline increases another molecule might be binding at a different site. This shows that the binding affinities of the two molecules of *o*-phenanthroline with XO are different.

References

1 R. C. Bray, in *The Enzymes*, ed. D. P. Boyer, Academic Press, New York, 1975, vol. XII, part B, p. 300.
2 R. Hille, V. Massey and T. G. Spiro (Editors), *Molybdenum Enzymes*, John Wiley & Sons, New York, 1985, pp. 443–518.
3 R. C. Bray, *Q. Rev. Biophys.*, 1988, **21**, p. 299.
4 R. Hille, G. N. George, M. K. Eidsness and S. P. Cramer, *Inorg. Chem.*, 1989, **28**, 4018.
5 H. Komai, V. Massey and G. Palmer, *J. Biol. Chem.*, 1969, **244**, 1692.
6 J. S. Olson, D. P. Ballon, G. Palmer and V. Massey, *J. Biol. Chem.*, 1974, **249**, 4350.
7 J. S. Olson, D. P. Ballon, G. Palmer and V. Massey, *J. Biol. Chem.*, 1974, **249**, 4363.
8 R. Cammack, M. J. Barber and R. C. Bray, *Biochem. J.*, 1976, **157**, 469.
9 R. Hille and V. Massey, 1991, **266**, 17401.
10 Zhi-Xing Wang, Hai-Bin Wu, Xi-Cheng Wang, Hai-meng Zhou and Chen-Lu Tsou, *Biochem. J.*, 1992, **281**, 285.
11 C. T. Privalle, S. E. Kong and I. Fridovich, *Proc. Natl. Acad. Sci. USA*, 1993, **90**, 2310.

12 V. Massey, P. E. Brumby, H. Komai and G. J. Palmer, *J. Biol. Chem.*, 1969, **244**, 1682.
13 M. S. Mondal and S. Mitra, *Biochemistry*, 1994, **33**, 10305.
14 M. S. Mondal and S. Mitra, *J. Inorg. Biochem.*, 1996, **62**, 271.
15 D. Edmondson, V. Massey, G. Palmer and L. M. Beachan, *J. Biol. Chem.*, 1972, **247**, 1597.
16 U. Penez and H. B. Dunford, *Biochemistry*, 1990, **29**, 2757.
17 R. J. Maguire and H B. Dunford, *Biochemistry*, 1972, **11**, 937.
18 H. Beinert, W. H. Orme-Johnson and G. Palmer, *Methods Enzymol.*, 1978, **54**, 111.
19 G. Palmer and V. Massey, *J. Biol. Chem.*, 1969, **244**, 2614.

Paper 8/07011C