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

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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. The reaction of XO with the 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.4–6 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 to 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.3 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-1H-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 aminocylase function.10–11 It has been shown that o-phenanthroline induces the soxRS regulon anaerobically by stimulation of soxS transcription.12 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) 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_2EDTA 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 anaerobic cuvette that was fitted with rubber septa. All spectrophotometric and CD
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 this absorbance at this wavelength in the presence of o-phenanthroline or 1,7-dimethylxanthine is due to the anaerobic reduction with 1,7-dimethylxanthine and 1,7-dimethylxanthine. The decrease in the absorbance at 450 nm due to the anaerobic reduction with 1,7-dimethylxanthine is observed for the reaction of horseradish peroxidase and lactoperoxidase with these substrates. The observed rate constants for the reaction of these two substrates with the enzyme. 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 complex formed between the oxidised form of XO and o-phenanthroline; \( k_1 \) and \( k_2 \) are the apparent second order rate constants for the formation of products; \( k_3 \) and \( k_{-3} \) are the forward and backward rate constants for the formation of \( (XO)_{ox-phen} \) complex from XO and phenanthroline. Eqn. (1) for \( k_{obs} \) can be derived from Scheme 1.

\[
k_{obs} = k_1[S] + \frac{k_3[S][S] + (k_3[S] + k_{-3})^{-1}}{1 + [k_3[S][S] + k_{-3}]^{-1}}
\]

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: \( k_1 = 1.6 \text{ M}^{-1} \text{ min}^{-1} \), \( k_2 = 123 \text{ M}^{-1} \text{ min}^{-1} \), \( k_{-2} = 18 \text{ min}^{-1} \) and \( k_3 = 104 \text{ M}^{-1} \text{ min}^{-1} \).

The observed rate constant \( k'_{obs} \) 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),
where 1,7-dimethylxanthine forms a reversible complex with XO leading to the formation of product.

Eqn. (2) for the observed rate constant \( k_{\text{obs}} \) can be derived for the above mechanism, where, \( S \) is 1,7-dimethylxanthine, \( k'_{\text{D}} = k'_{\text{D}} + k'_{\text{S}} \) and \( k'_{\text{S}} \) 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 \( k'_{\text{D}} \) and \( k'_{\text{S}} \) obtained from

\[
k'_{\text{obs}} = \frac{k'_{\text{D}}[S]}{k'_{\text{D}} + [S]} \tag{2}
\]

such a fit are 0.08 \( \text{min}^{-1} \) and 440 \( \mu \text{M} \) respectively. The results indicate a weak binding affinity of 1,7-dimethylxanthine with XO.

**Circular dichroism study**

Xanthine oxidase displays a rather intense CD spectrum, which strongly resembles that of spinach ferredoxin and other Fe/S proteins. 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 \( k_{\text{obs}} \). A typical value of \( k_{\text{obs}} \) was 0.01 min\(^{-1}\) at 4 mM concentration of o-phenanthroline. Fig. 3B shows the variation of \( k_{\text{obs}} \) 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, \( k_{\text{obs}} \) was 0.01 min\(^{-1}\) at 11.5 \( \mu \text{M} \) 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 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),

\[
A_{450} = A_{450}^{\text{FAD}} \frac{E_{450}^{\text{FAD}}}{E_{550}^{\text{FAD}}} \times A_{550} \tag{3}
\]

where \( A_{450} \) and \( A_{550} \) are the absorbances at 450 nm and 550 nm respectively; \( E_{450}^{\text{FAD}} \) 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 \( k_{\text{obs}} \). A typical value of \( k_{\text{obs}} \) obtained from such an exponential fit was 0.039 min\(^{-1}\) at 4 mM concentration of o-phenanthroline. The values of \( k_{\text{obs}} \) 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, \( K'_{\text{D}} = \frac{k'_{\text{D}}}{k'_{\text{S}}} \) and \( k'_{\text{S}} \) is the rate constant for the formation of product from the complex.

\[
\text{FAD} + \text{phen} \xrightarrow{k'_{\text{obs}}} \text{FAD} - \text{phen} \xrightarrow{k'_{\text{S}}} \text{Products}
\]

Scheme 3

For \( k_{\text{obs}} \), eqn. (4) can be derived from the above mechanism.
The double reciprocal plot of 1/\( \frac{k_{2,obs}}{t} \) vs 1/[phen] from such an exponential function, which gave the observed rate constants. A typical value of the observed rate constant (\( k'_{2,obs} \)) from such an exponential fit was 0.03 min\(^{-1}\) at 196 \( \mu \)M 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 \( k''_{2} \) obtained from this method are 0.06 min\(^{-1}\) and 228 \( \mu \)M respectively.

**References**


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