

## Altered redox affinity of xanthine oxidase active sites by copper(II) ions

MADHU SUDAN MONDAL and SAMARESH MITRA\*

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

**Abstract.** The interaction of  $\text{Cu}^{2+}$  ion with the redox centres of xanthine oxidase (XO) has been investigated using optical difference spectroscopic measurements. Anaerobic enzyme-reduction experiments using controlled and excess substrates (xanthine and NADH) have been performed to investigate the perturbation of XO active sites in the presence of  $\text{Cu}^{2+}$  ions. The results indicate an overall alteration in the redox affinities (i.e. affinity to accept electrons) of the active sites of XO by  $\text{Cu}^{2+}$  ion.

**Keywords.** Xanthine oxidase; redox reaction;  $\text{Cu}^{2+}$ -XO interaction.

### 1. Introduction

Milk xanthine oxidase (xanthine: oxygen oxidoreductase, EC 1.2.3.2.) is a complex metallo-flavo enzyme. It catalyses the oxidation of xanthine to uric acid in the presence of oxygen and during this process oxygen is reduced to either hydrogen peroxide or superoxide radical. Xanthine oxidase (XO) has a wide specificity towards substrates and reacts with other reducing substrates such as nicotinamide adenine dinucleotide (NADH). It has two independent subunits each containing four redox centres: a molybdenum (VI) centre, two iron-sulfur (Fe/2S) clusters called Fe/S I and Fe/S II and a flavin adenine dinucleotide (FAD) unit<sup>1-6</sup>. During the catalytic reaction, the molybdenum (VI) and the FAD centres accept two electrons each while the iron-sulfur clusters accept one electron each from the substrate (i.e. a total of six electrons per FAD unit of the enzyme molecule). Once electrons are introduced within the enzyme (using substrates such as xanthine, NADH, etc), there is fast electron transfer process to reach equilibrium-electron-distribution among the four redox centres of the enzyme. FAD and Fe/S centres show characteristic absorption bands in the visible region. The intensities of these bands decrease upon addition of electrons to the enzyme from substrate, and this decrease has earlier been used as an indirect measure of the level of equilibrium-electron-distribution at each redox centre<sup>7-10</sup>. When both xanthine and oxygen react with XO, the redox centres of the enzyme undergo a dynamic inter-conversion between their oxidised and reduced states, and, therefore, the level of equilibrium-electron-distribution cannot be monitored under such conditions. However, these measurements can be carried out by reducing the enzyme with substrates (e.g. xanthine) under anaerobic condition (i.e. no oxygen present).

Various inorganic and organic molecules are known to interact with XO and affect the potentials of individual redox centres. For example, the interaction of arsenite with XO

---

\*For correspondence

was found to increase the Mo(VI) reduction potential<sup>11</sup>. Clearly, such interactions would either activate or inhibit the function of the enzyme. XO has been observed to be inhibited by a range of inorganic and organic molecules. In recent times, various metal ions have been found to inhibit the activity of XO<sup>12-14</sup>. Liochev *et al*<sup>15</sup> investigated the interaction of vanadate ion on the oxidation of NADH by XO. Cu<sup>2+</sup> ion has been considered to be responsible for the decrease in XO activity in neoplastic cells<sup>16-17</sup> and reported to inhibit the solution activity of XO<sup>12,18,19</sup>. However, the effects of Cu<sup>2+</sup> ion on the individual redox centres of XO are unknown.

In the present paper, we describe the results on the equilibrium electron distribution in native and Cu<sup>2+</sup>-inhibited enzyme. Two independent reduction (controlled and excess-substrate) experiments of XO have been performed using xanthine and/or NADH as electron donor. The results from these two experiments suggest perturbation of the redox chromophores of XO by Cu<sup>2+</sup> ion.

## 2. Experimental

Fresh unpasteurized cow's milk was obtained from Aarey Milk Colony, Goregaon, Bombay. Xanthine oxidase (XO) was isolated by the reported procedure<sup>20</sup>. 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 of 90–110 which corresponds to ~40–50% functional enzyme<sup>21</sup>. The concentration of the enzyme was determined spectrophotometrically by using the molar extinction coefficient of the enzyme as 37,800 M<sup>-1</sup> cm<sup>-1</sup> at 450 nm<sup>20</sup>.

Analytical grade copper(II) chloride and buffers were used. The EDTA used for the isolation of the XO was removed by dialysing it thoroughly against the buffer. In all the experiments millipore milli-Q quantity water was used. The experiments were performed in 50 mM NaH<sub>2</sub>PO<sub>4</sub> buffer. A stock of 1 M buffer was prepared, and metal ions from this buffer were removed by passing it through Bio Rex ion exchange resin. Diluted buffer, whenever necessary, was prepared from this stock buffer by diluting it with milli-Q water. The Cu<sup>2+</sup>-inhibited XO was prepared by incubating the enzyme with CuCl<sub>2</sub> for at least 30 min.

All spectrophotometric experiments were performed on Shimadzu UV-2100 spectrophotometer. The anaerobic titration was carried out in an anaerobic cuvette which was fitted with rubber septa. The sample was first made anaerobic by purging high quality argon gas for 15 min. The argon gas were continued to pass above the surface of the anaerobic sample while the experiments were in progress to make sure complete anaerobic atmosphere. The pH of the sample was measured by a pH meter from Orion Research.

## 3. Results

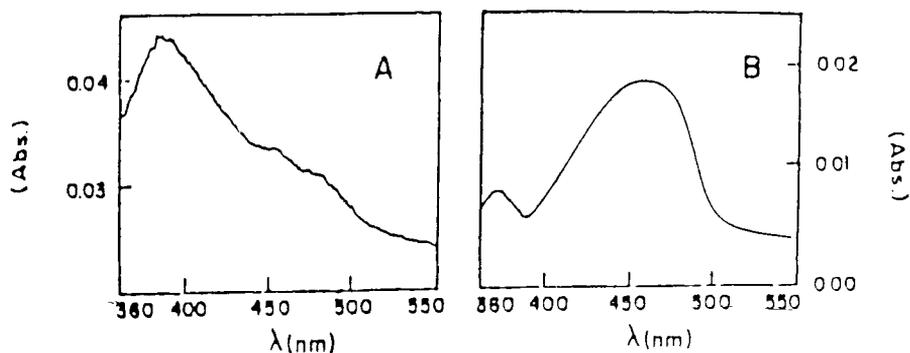
### 3.1 Controlled-substrate reduction of XO

XO was partially reduced under anaerobic condition by controlled addition of NADH. Under the condition that [XO] >> [NADH], only one molecule of substrate can bind to XO. Since NADH transfers two electrons, all NADH-bound XO molecules would form two-electron reduced enzyme. However, the transfer of these two electrons is known to be slow and requires 20–30 min for the completion of this process<sup>22</sup>. In the present situation, we have therefore performed partial reduction experiments on native and Cu<sup>2+</sup>-inhibited

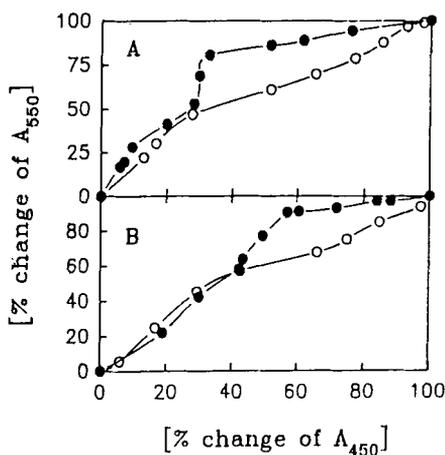
enzymes by NADH, and measured the optical spectra of these two enzymes before and after their reduction (data not shown). The optical spectra of the partially reduced enzyme was recorded after 30 min of the addition of NADH. In order to quantify the change in absorbance due to reduction, the optical spectra of the native and  $\text{Cu}^{2+}$ -inhibited enzyme were subtracted from their corresponding partially-reduced optical spectra. Figures 1A and 1B show the difference spectra obtained from such subtraction procedure and represents respectively  $\text{Cu}^{2+}$ -inhibited and native enzyme. The difference spectra of native enzyme has earlier been reported<sup>22</sup>, which is similar to that obtained in this experiment. The spectrum for the native enzyme shows a broad absorption maxima near 450 nm. This type of broad maximum has earlier been shown to be a signature for the reduction of FAD and Fe/S centres of the enzyme. However, it is evident from the figure that the nature of the difference spectra of native and  $\text{Cu}^{2+}$ -inhibited enzyme are different and the broad absorption maximum at 450 nm is absent in case of  $\text{Cu}^{2+}$ -inhibited enzyme. This indicates that a major fraction of the electrons of two-electron reduced enzyme is not associated with FAD or Fe/S centres, but with the molybdenum centre (see also the discussion section).

### 3.2 Excess-substrate reduction of XO

Anaerobic reduction of XO in presence of excess substrates (such as xanthine and NADH) can be used to detect the order of redox affinity (affinity to accepting electrons) of the active sites of XO. Under the condition that  $[\text{xanthine}] \gg [\text{XO}]$ , xanthine initially introduces a major percentage of its electrons to a centre with the highest redox affinity. As the concentration of xanthine increases, more electrons are added to the enzyme and all the redox centres are gradually saturated with electrons according to their order of redox affinity. A gradual addition of electrons to the enzyme can be easily detected by



**Figure 1.** The optical difference spectra of two-electron reduced  $\text{Cu}^{2+}$ -inhibited (A) and native (B) at pH 7.6 in 50 mM  $\text{NaH}_2\text{PO}_4$  buffer. The optical spectra of native  $\text{Cu}^{2+}$ -inhibited were recorded both before and after addition of NADH under anaerobic condition. The difference spectra were obtained by subtracting optical spectra of native and  $\text{Cu}^{2+}$ -inhibited enzyme from their corresponding partially reduced spectra. The concentration of XO was 9.2 mM. The concentration of  $\text{Cu}^{2+}$  ion in the inhibited enzyme was 6.5  $\mu\text{M}$ . The NADH concentrations were 2.5 and 4.5  $\mu\text{M}$  for native and inhibited enzymes respectively.



**Figure 2.** The percentage (%) change of absorbance ( $\Delta A$ ) at 450 and 550 nm due to the reduction of the enzyme by xanthine (A) at pH 7.3 and NADH (B) at pH 7.6. Hollow and filled symbols represent the titration for native ( $1.8 \mu\text{M}$ ) and  $\text{Cu}^{2+}$ -inhibited enzyme ( $19.5 \mu\text{M}$ ) respectively. Solid lines show the trend of the data points. The concentration of  $\text{Cu}^{2+}$  ion in the inhibited enzyme was  $65 \mu\text{M}$ . % $\Delta A$  at a particular wavelength was determined from the ratio of  $\Delta A$  at certain concentration of substrate to the maximum  $\Delta A$  observed at excess concentration of the substrate. When the enzyme was reduced anaerobically by xanthine, the optical spectra were recorded immediately and after 20 min of the addition of anaerobic xanthine. Almost identical results were obtained in the two experiments. In the case of reduction by NADH, the optical spectrum of the enzyme was recorded after 25 min of successive additions of anaerobic NADH.

measuring the decrease in optical spectra of the enzyme. It is known that both FAD and Fe/S centres of XO contribute to the absorption at 450 nm, while at 550 nm, only the Fe/S centres absorb<sup>20</sup>. Hence, a plot of percentage change in absorbance at 450 nm vs 550 nm would be useful to detect the order of redox affinity of the active sites of the enzyme<sup>10</sup>.

We have therefore performed reduction experiments with increasing concentration of xanthine and measured the decrease in absorbance at 450 nm and 550 nm at each xanthine concentration. These data have been used to obtain the percentage change in absorbance at 450 and 550 nm and plotted in figure 2 (upper panel). Xanthine introduces electrons at the molybdenum centre<sup>23</sup>; whereas NADH introduces electrons at the FAD centre. In order to check the role of the electron introduction site on equilibrium electron distribution, we have performed similar reduction experiments for native and  $\text{Cu}^{2+}$ -inhibited enzyme with NADH as substrate. These data have also been used to determine percentage change in absorbance at 450 and 550 nm and are shown in figure 2 (bottom panel).

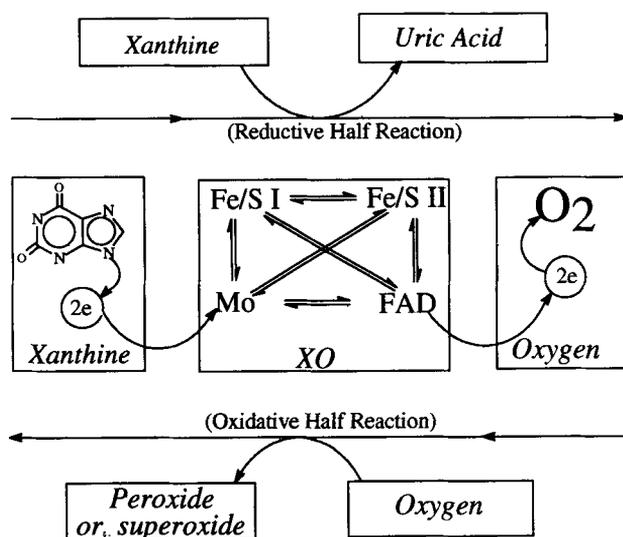
It is evident from the figure that the corresponding titration curves for xanthine and NADH are similar. Thus this result indicates that the site of introduction of electrons does not have any significant role in determining the electron equilibration within the redox centres. However, the results indicate that the reduction curves for native and inhibited enzymes are significantly different (see figure 2).

## 4. Discussion

### 4.1 General description of the catalytic reaction of XO

As depicted in scheme 1, the complete analytic cycle of this enzyme consists of two half reactions which are known as reductive half-reaction (RHR)<sup>7,24-26</sup>, and oxidative half-reaction<sup>27</sup>.

During RHR, xanthine reacts with XO to reduce (i.e. electrons are added to enzyme) the oxidised redox-centres of the resting-state enzyme. Xanthine is known to bind at the molybdenum atom of XO<sup>23</sup> and hence the reduction of the enzyme begins at the molybdenum centre. During OHR, oxygen reacts with the reduced-redox-centres of XO to oxidise (i.e. electrons are removed from the enzyme) the enzyme to its resting-state. Oxygen is known to bind at the FAD centre of XO and thus the oxidation of the enzyme begins at the FAD centre<sup>27</sup>. During the process of RHR, there is a fast equilibration process of electrons (which starts at the molybdenum centre) among the four redox centres of XO. This equilibrium process has been described by a rapid equilibrium model<sup>7</sup>. According to this model, the equilibrium-electron-distribution at each redox centre depends on the corresponding reduction potentials of the four centres, and the redox affinity of each centre is independent of the number of reducing equivalents accepted by the enzyme molecule. Therefore, any change in equilibrium-electron-distribution (we have measured this equilibrium indirectly by monitoring the changes in optical spectra upon addition of substrate) in presence of  $\text{Cu}^{2+}$  ion is a measure of the perturbation of the redox affinity of the active sites of XO. The reduction potentials of native XO active sites have been determined by calorimetric technique and these values are:  $-197 \text{ mV (Fe/S II, } 1e^-) > -235 \text{ mV (FAD } 2e^-) > -300 \text{ mV (Fe/S I, } 1e^-) > -335 \text{ mV (Mo, } 1e^-)$  at pH 7.2<sup>28</sup>. It is noted here that more positive reduction potential of a centre indicates its high redox affinity.



Scheme 1.

#### 4.2 Controlled-substrate reduction of XO

Controlled-substrate reduction experiments were performed by adding only two electrons to the enzyme (total six electrons are required for complete reduction of the enzyme). Under this condition, a considerable fraction of these two electrons will be associated with the redox centres with higher redox affinities. According to the reported potentials of the redox centres of native XO (see above), the order of the redox affinity is Fe/S II > FAD > Fe/S I > Mo(VI). Thus, when only two electrons are added to the enzyme, a major fraction of these electrons will preferably reside at the Fe/S or FAD centres, and not with molybdenum centre. The Fe/S and FAD centres are known to show a broad absorption maximum at 450 nm, while molybdenum centres do not have appreciable absorbance in the visible region. Therefore, the observation of a broad maximum near 450 nm in the difference spectra (i.e. reduction of Fe/S and FAD centres) of native enzyme is consistent with the redox affinities of the active sites. In contrast, the absence of a broad absorption maximum at 450 nm for the Cu<sup>2+</sup>-inhibited enzyme (see figure 1B) indicates that a certain fraction of two-electrons is associated with a centre that is other than FAD or Fe/S centres. We therefore suggest that some fraction of the two-electron are used to reduce the molybdenum centre and that the redox affinity of this centre is increased in presence of Cu<sup>2+</sup> ion. It should be also noted that the redox affinity of the molybdenum centre is the least among all other centres in native XO. Thus, the observation of electrons going to the molybdenum centre, even for two-electron reduced Cu<sup>2+</sup>-inhibited enzyme, must be associated with the strong perturbation of the molybdenum centre by Cu<sup>2+</sup> ion.

#### 4.3 Excess-substrate reduction of XO

During the initial reduction of native enzyme, the electrons preferentially reduce the 550 nm band as compared to 450 nm band (see figure 2, top panel). In other words, at the beginning of reduction, electrons preferentially reduce Fe/S centres rather than the FAD center. After about 50% reduction of the 550 nm band, preferential reduction of the 450 nm band could be observed. All these results are consistent with the order of redox affinity (Fe/S II > FAD > Fe/S I > Mo(VI)) of the native enzyme. The results indicate initial reduction of Fe/S centre which is consistent with the order of redox affinity for Fe/S II. Once this centre tends towards saturation, electrons preferentially reside at the FAD centre (not Fe/S I) and reduces the 450 nm band.

Following a similar analogy for the Cu<sup>2+</sup>-inhibited enzyme, we observe that initial 50% reduction of 550 nm band is similar to that of native enzyme. This indicates that, similar to the native enzyme, initial electrons preferentially reside at the Fe/S II centre. However, in the latter part of the curve, we are unable to observe preferential reduction of the 450 nm band. The data rather indicates, unlike native enzyme, preferential reduction of the 550 nm band i.e. reduction of Fe/S centre. Both Fe/S I and Fe/S II have the highest redox affinity, initial electrons will mainly reduce this centre. Hence, the final 50% reduction of the 550 nm band would arise mainly from Fe/S I centre. It is therefore suggested that, in the Cu<sup>2+</sup>-inhibited enzyme, the redox affinity of the Fe/S I centre is higher than the FAD centre.

Further evidence in support of the above suggestions are obtained from the reported mean distances of the active sites of XO<sup>29-31</sup>. It has been observed that the distance between Mo and Fe/S I is closest among all other distances between the redox centres.

Thus Mo and Fe/S I centres are the nearest 'neighbours' within the enzyme. Our results of the two-electron reduced enzyme indicate that Cu<sup>2+</sup> ion increases the redox affinity of the molybdenum centre. Since Fe/S I is close to the Mo centre, the redox affinity of this centre is also expected to be altered in presence of Cu<sup>2+</sup> ion.

## 5. Conclusions

Two important questions concerning the interaction of Cu<sup>2+</sup> ion with XO have been addressed in this work. Does Cu<sup>2+</sup> ion alter the redox affinity of the active sites of XO? And whether such an effect, if any, can be quantified? The answer to the first question is yes. Our results indicate an overall alteration in the redox affinity of the active sites. However, the order of this alteration can only be partially quantified from Fe/S II > FAD > Fe/S I > Mo(VI) in the native enzyme to Fe/S II > Fe/S I > FAD in Cu<sup>2+</sup>-inhibited enzyme. The redox affinity of the Mo centre is greatly enhanced in the presence of Cu<sup>2+</sup> ion and is expected to be close to that of Fe/S I centre. Controlled substrate experiments are performed with two-electron reduced enzyme and this allows us to 'locate' where (i.e. molybdenum centre) a considerable fraction of these two-electrons resides in Cu<sup>2+</sup>-inhibited enzyme. Similarly, the results from the experiments of excess-substrate reduction have been used to determine the order of redox affinity of active sites of XO in presence of Cu<sup>2+</sup> ion.

Thus this manuscript describes the effects of an inhibitor with the individual active sites of a complex metalloenzyme and provides an indirect method to monitor alteration in redox affinity of the active sites by using the enzyme's own physiological substrate (e.g. xanthine) as a probe. The electron-equilibrium within the redox centres of the native enzyme appears to be consistent with their corresponding reduction potential. Thus the results once again support the previously documented idea<sup>7</sup> that the equilibrium-electron-distribution among the redox centres of XO are determined only by their intrinsic redox affinity and that the equilibrium process does not depend on the site of introduction of electrons.

## References

1. Bray R C 1975 In *The enzymes* (ed.) D P Boyar (New York: Academic Press) vol 9, part B, pp 300–419
2. Hille R and Massey V 1985 In *Molybdenum enzymes* (ed.) T G Spiro (New York: John Wiley and Sons) pp 443–518
3. Bray R C 1988 *Q. Rev. Biophys.* **21** 299
4. Kramer S P, Johnson J L, Rebeiro A A, Millington D S and Rajagopalan K V 1987 *J. Biol. Chem.* **262** 16357
5. Wilson G L, Greenwood R J, Pilbrow J R, Spence J T and Wedd A G 1991 *J. Am. Chem. Soc.* **113** 6803
6. Skibo E B, Gilchrist J H and Lee C H 1987 *Biochemistry* **26** 3032
7. Olson J S, Ballou D P, Palmer G and Massey V 1947a *J. Biol. Chem.* **249** 4350
8. Hille R, Hagen W R and Dunham W R 1985 *J. Biol. Chem.* **260** 10569
9. Stewart R C, Hille R and Massey V 1984 *J. Biol. Chem.* **259** 14426
10. Hille R, Fee J A and Massey V 1981 *J. Biol. Chem.* **256** 8933
11. Hille R, Stewart R C, Fee J A and Massey V 1983 *J. Biol. Chem.* **258** 4849
12. Mondal M S, Behere D V and Mitra S 1994 *Proc. Indian Acad. Sci (Chem. Sci.)* **106** 1547; Sau A K, Mondal M S and Mitra S 1998 *J. Chem. Soc., Dalton Trans.* 4173
13. Sau A K, Behere D V and Mitra S 1994 *J. Chem. Soc., Chem. Comm.* 1547
14. Mondal M S and Mitra S 1996 *J. Inorg. Biochem.* **112** 65

15. Liochev S, Ivancheva E and Fridovich I 1989 *Arch. Biochem. Biophys.* **269** 188
16. Prajda N, Morris H P and Weber G 1976 *Cancer Res.* **36** 4639
17. Weber G 1973 *Adv. Enzyme Regul.* **11** 79
18. Bergel F and Bray R C 1959 *Biochem. J.* **73** 182
19. Kela U and Vijayvargiya R 1981 *Biochem. J.* **193** 799
20. Massey V, Brumby P E, Komai H and Palmer G 1969 *J. Biol. Chem.* **244** 1682
21. Edmondson D, Massey V, Palmer G and Beachan L M 1972 *J. Biol. Chem.* **247** 1597
22. Stewart R C, Hille R and Massey V 1984 *J. Biol. Chem.* **259** 14426
23. Hille R, George G N, Eidsness M K and Cramer S P 1989 *Inorg. Chem.* **28** 4018
24. Hille R and Massey V 1991 *J. Biol. Chem.* **266** 17401
25. McWhirter R B and Hille R 1991 *J. Biol. Chem.* **266** 23724
26. Mondal M S and Mitra S 1994 *Biochemistry* **33** 10305
27. Olson J S, Ballou D P, Palmer G and Massey V 1974b *J. Biol. Chem.* **249** 4350
28. Porras A G and Palmer G 1982 *J. Biol. Chem.* **257** 11617
29. Coffman R E and Beuttner G R 1979 *J. Phys. Chem.* **83** 2392
30. Coughlan M P, Betcher-Lange S L and Rajagopalan K V 1979 *J. Biol. Chem.* **254** 10694
31. Nagler L G and Vartanyan L S 1976 *Biochim. Acta* **427** 78