Proc. Indian Acad. Sci. (Chem. Sci.), Vol. 106, No. 1, February 1994, pp. 29–35.
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Inhibition of oxidoreductase activity of xanthine oxidase by Cu^{2+} and Hg^{2+} ions

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MS received 3 September 1993; revised 28 December 1993

Abstract. Xanthine oxidase has been isolated in good yield and pure form. Inhibition of the enzyme by Cu^{2+} and Hg^{2+} ions has been studied. The nature and extent of the inhibition have been determined.

Keywords. Xanthine oxidase; inhibition of XO; interaction of metal ion with XO.

1. Introduction

Bovine xanthine oxidase (xanthine: oxygen oxidoreductase, EC 1.2.3.2.) catalyses the oxidation of xanthine to uric acid in the presence of oxygen. It is a complex metallo-flavo enzyme with two independent subunits each containing one molybdenum(VI) centre, two iron-sulphur (2Fe/2S) clusters called Fe/S I and Fe/S II, and one FAD (flavin adenine nucleotide) unit (Bray 1975, 1988; Hille and Massey 1985). The molybdenum co-factor consists of a molybdenum pterin ring, and the molybdenum atom is bonded to an 'S' atom by a double bond (Mo=S). The presence of 'S' has been shown to be catalytically important (Kramer *et al* 1987; Wilson *et al* 1991). During the oxidation of xanthine to uric acid, xanthine binds to the molybdenum atom of the enzyme and the electron transfer from substrate to the enzyme takes place at this centre (Hille *et al* 1989). Xanthine oxidase (XO) is one of the well-studied molybdenum-containing enzymes. Isolation and purification of this enzyme from milk have been reported by many authors (Hart and Bray 1967; Massey *et al* 1969: Nishino *et al* 1981; Ventom *et al* 1988; Hunt and Massey 1992). As the composition of milk varies in different laboratories, attempts to isolate this enzyme using reported procedures sometimes lead to poor yields. It is, therefore, necessary to assess the critical steps in the isolation procedure, which needs modification due to variation in the composition of milk.

Metal ions play an important role in regulating the functions of many biological molecules. It is known that Cu^{2+} ions play an important role in many biological processes and control the syntheses of proteins in higher plants in growth media (Howell 1958; Kamamoto *et al* 1973; Evans 1979; Zhang *et al* 1992). Mercury shows

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very high permeability through the lipid bilayer membrane which indicates its ability to enter inside tissue cells (Karniski 1992). XO is found inside the mammalian epithelial and capillary endothelial cells. The interactions of these metal ions with XO inside the cells may therefore have significant effect in controlling tissue damage and purine catabolism by the enzyme. It has been reported that the interaction of Cu^{2+} ion towards XO shows an inhibitory effect (Bergel and Bray 1959; Kela and Vijayvargiya 1981). Kela and Vijayvargiya (1981) reported 96·7% inhibition of the enzyme by Cu^{2+} ion. The authors used a colorimetric method to assay the enzyme by using triphenyltetrazolium chloride at pH 7·4. Gardlik and Rajagopalan (1990) have shown that Hg^{2+} ions block the terminal sulphur atoms of the molybdenum centre of XO but their effect on enzyme activity has not been reported. As with Cu^{2+} ions, the interaction of Hg^{2+} ions with the enzyme may also be inhibitory.

In the present paper, we briefly report the isolation of xanthine oxidase. The inhibition of the enzyme by Cu^{2+} and Hg^{2+} ions has been investigated.

2. Materials and methods

All reagents used were of analytical grade. Pancreatin was obtained from pig pancrease. Xanthine and α-cellulose were purchased from the Sigma Chemical Co, USA. Whatman product CF11 was used to prepare the column matrix for the purification of the enzyme.

XO was isolated and purified following some modification of the reported procedure (Massey *et al* 1969). Fresh unpasteurised cow's milk was obtained from the Aarey Milk Colony, Goregaon, Bombay. Cream (2·2 kg) was extracted from this fresh milk (30 l) by centrifugation. It was suspended in 10 mM sodium phosphate buffer to make a total volume of about 3·5 litres. The homogeneous suspension was treated with sodium salicylate (0·2 g/l), cysteine hydrochloride (0·3 g/l), EDTA (0·37 g/l), $NaHCO_3$ (3 g/l), and was stirred vigorously to make a homogeneous mixture. Pancreatin (1·6 g/l) was added to it and the mixture was stirred for 4 hours at 30°C, and left overnight at 4°C. The XO-bound fat particles of the digest were solubilised and brought into the aqueous layer by the ammonium sulphate-butanone fractionation method. This step was found to be critical for obtaining the enzyme in high yields. In the reported procedure, 168 ml of butanol and 190 g of ammonium sulphate were used for one litre of the digest. Our experiments with various concentrations of ammonium sulphate show that about 130 g of this salt per litre of the digest gave optimum yield and purity of the enzyme. After this fractionation, the digest was centrifuged and the lower aqueous layer collected. The aqueous layer which contains XO was again fractionated by ammonium sulphate (170 g per litre) to precipitate the enzyme. The precipitated protein was centrifuged and suspended in minimum volume of 100 mM phosphate buffer. It was dialysed against the same buffer containing 0·3 mM EDTA and 1 mM sodium salicylate for 24 hours. The purification of the enzyme was performed by the reported calcium phosphate–cellulose column chromatography (Massey *et al* 1969). It was performed in a large column (8 × 50 cm) which was connected to an Isco WIZ^{TM} peristaltic pump, FOXY^{TM} fraction collector and UA-5 absorbance detector. The calcium phosphate–cellulose column matrix was prepared according to a previous report (Swingle and Tiselius 1951). We have used two types of cellulose (CF11 and α-cellulose) for making the column matrix. It was found that both types of cellulose

can be used for the purification of the enzyme. The absorbance of the eluate from the column was monitored continuously at 280 nm by the UA-5 detector. The purification of the enzyme after removing the impurities has been shown in figure 1 where the removal and purification of the impurities are represented by 1 and 2. The concentrated and purified enzyme was obtained by further ammonium sulphate fractionation. The optical spectrum of the purified XO is shown in figure 2 (curve 1) which is almost identical to the reported spectrum (Massey *et al* 1969). The AFR (activity-to-flavin ratio) value of the purified enzyme was in the range of 130–140, which corresponds to 59 to 64% of the active form of the enzyme (Edmondson *et al*

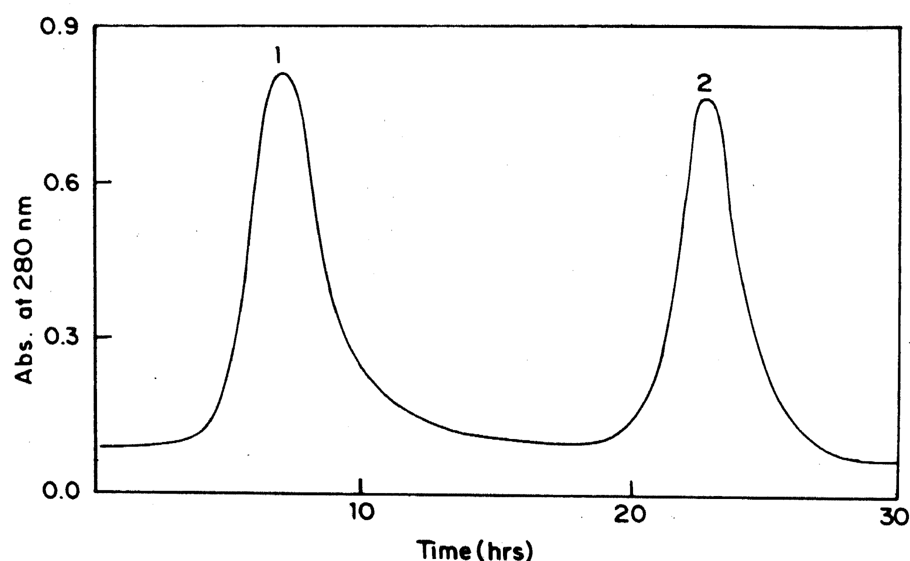

Figure 1. The response of the UA-5 absorbance detector monitored at 280 nm showing the purification of the enzyme. The first peak (1) corresponds to the removal of the impurities.

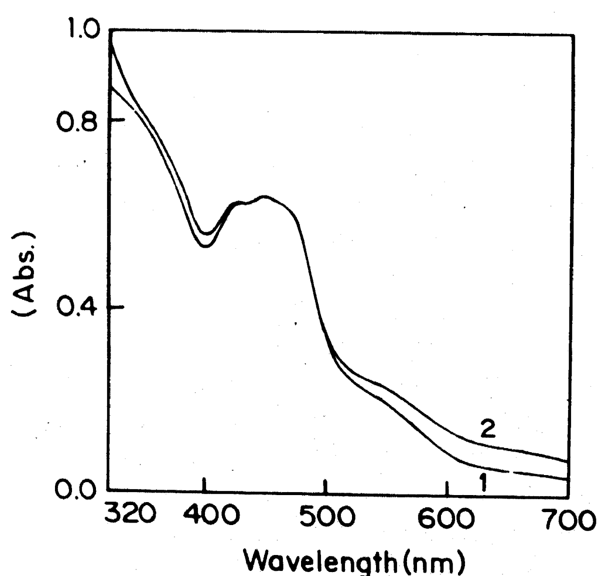

Figure 2. The optical spectra of the native (17 μM, curve 1) and Cu^{2+} treated XO (0·5 mM, curve 2).

1972). The PFR (protein-to-flavin ratio) value of the purified enzyme was 5·6 which is close to the reported value of 5·4 (Massey *et al* 1969). These results together with the optical spectrum in figure 2 establish the identity and purity of the enzyme.

The activity of the enzyme was measured spectrophotometrically by monitoring the formation of uric acid from xanthine at 295 nm. The concentration of XO was determined spectrophotometrically by taking the molar extinction coefficient of the enzyme as 37,800 $M^{-1} cm^{-1}$ at 450 nm (Massey *et al* 1969). For studying the metal ion inhibition of the enzyme, the EDTA present in the purified XO was removed by dialysing it thoroughly against EDTA free buffer. In all the inhibition studies millipore milli-Q quality water was used. The experiments of the inhibition studies were performed with 10 mM NaH_2PO_4 buffer.

Metal ion-free enzyme was prepared by dialysing the enzyme against 1 mM EGTA for 12 hours in 10 mM NaH_2PO_4 buffer at $pH = 7{\cdot}0$. After this treatment, EGTA was removed by dialysis against the same buffer. A stock 1(M) buffer was prepared and metal ions were removed by passing through Bio Rex ion-exchange resin. Diluted buffer, whenever necessary, was prepared from this stock buffer by diluting it with milli-Q water. The inhibited enzyme was prepared by incubating XO with the metal ions.

3. Results and discussion

3.1 *Inhibition of XO activity by Cu^{2+} ion*

The interaction of Cu^{2+} ion with XO does not show any large change in the optical spectra of XO (figure 2) though some changes at $\lambda > 550$ nm are observed. The inhibition of XO oxidoreductase activity by Cu^{2+} ion has been examined by incubating the enzyme with different concentrations of the metal ion for at least one hour. The activity of the different mixtures was measured by monitoring the steady increase in absorbance at 295 nm for the formation of the product uric acid from xanthine. The Cu^{2+} ion is found to inhibit the enzyme activity, which is in agreement with previous studies (Kela and Vijayvargiya 1981). Figure 3 shows variation of inhibition of XO with the Cu^{2+} ion concentration in the range 0–165 μM. The data can not be fitted to a single exponential, which suggests the multiphasic nature of the interaction of Cu^{2+} ion with XO. Similar nature of inhibition/activation has been interpreted as a signature of multiphasic interaction (Bastelaere *et al* 1992; Bernardo *et al* 1993; Hochman *et al* 1993). The enzyme shows about 58% inhibition at $pH = 7{\cdot}2$ when incubated with 165 μM Cu^{2+} ion concentration which disagrees with the value of 96·7% reported earlier by Kela and Vijayvargiya (1981). According to their procedure, they used the conversion reaction of xanthine to uric acid catalysed by XO for the hydrogenation of colourless triphenyl tetrazolium chloride to red triphenyl formazan. Acetic acid was used to terminate the reaction after 5 minutes and the formation of formazan, monitored at 495 nm, was used for the activity calculation. The formation of uric acid was estimated in this indirect manner after 5 minutes of the start of the reaction. The condition of linearity, which is essential for the calculation of the activity, will not be valid for such a length of time in the presence of higher concentrations of Cu^{2+} ions. Thus, the rate measured from the formation of formazan after 5 minutes would underestimate the true activity of the enzyme giving an incorrect value of the

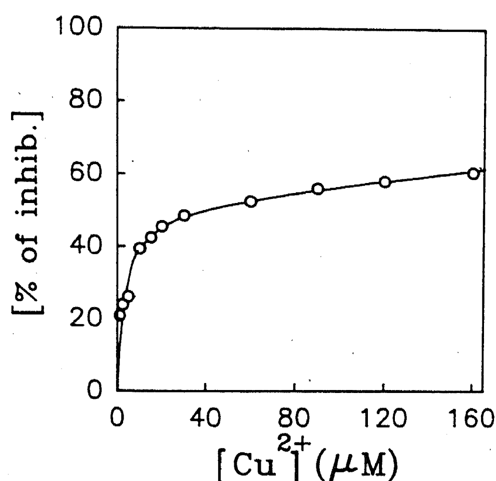

Figure 3. The dependence of the percentage of the inhibition of XO activity on the Cu^{2+} ion, at $pH = 7.2$ (○). The activity of the enzyme was measured spectrophotometrically by monitoring the formation of uric acid at 295 nm allowing 30 μM of xanthine to react with 6.6×10^{-8} M XO which was incubated with Cu^{2+} for at least one hour.

percentage of inhibition of the enzyme. In our measurements, we have directly monitored the conversion of xanthine to uric acid at 295 nm during the initial 20 seconds of the reaction, which ensures the condition of linearity of the activity measurement and hence our measurements are expected to yield a more accurate value of percentage inhibition.

During the first phase of the inhibition, almost 48% inhibition takes place when the enzyme is incubated at only 30 μM Cu^{2+} ion concentration. The scatter in the data at very low Cu^{2+} concentration (1–5 μM) was due to errors in measurement of the metal ion concentration. The difference between the total and first phase of the inhibition is ~ 10%. The multiphasic nature of the inhibition may arise due to the binding of Cu^{2+} ions to different sites of the enzyme. This may also occur due to the existence of the time dependence of the metal-binding equilibrium. In the present case, however, we have incubated the enzyme for sufficient time so that the time-dependent effect may not have any contribution in the steady-state inhibition of the enzyme. This was confirmed by the observation that the percentage of inhibition remained constant after the total incubation period. The multiphasic nature of the inhibition may therefore arise from at least two different types of binding of the metal ion to the enzyme. The multiphasic binding of metal ions to protein molecules is known in biological systems. The binding of metal ions to D-xylose isomerase was found to give multiphasic steady-state activation of the enzyme (Bastelaere *et al* 1992). The results of the binding in the present case may be rationalised considering the complex structure of XO. The active site of XO consists of four redox centres which are bonded to inorganic and organic 'S' and 'N' donor atoms. The first phase of the inhibition, which is responsible for the major decrease of the enzyme activity, must be due to the binding of Cu^{2+} ions near the active site. The remaining percentage of the inhibition may arise from the binding of Cu^{2+} to different amino acids of the protein by electrostatic or other nonspecific forces, which have some degree of regulatory effects on XO activity. The Cu^{2+} ion is known to have high affinity for binding to 'S' donor atoms. XO has an inorganic S atom in its molybdenum co-factor

which plays an important role in its catalytic pathway. There is a possibility that the Cu^{2+} ion may interact with the S atom of the molybdenum co-factor which may lead to the inhibition of the enzyme. However, detailed kinetic studies are needed to establish this possibility, which are currently underway.

3.2 *Inhibition of XO by Hg^{2+} ions*

Incubation of Hg^{2+} ions with XO decreases the activity of the enzyme. The inhibition has been investigated spectrophotometrically as in the case of Cu^{2+} ions. The enzyme was incubated for at least half an hour at different concentrations of Hg^{2+} ions for steady state activity measurements. Figure 4 shows the dependence of percentage of inhibition on Hg^{2+} concentration in the range 0·0 to 60·0 μM at pH = 7·2. The steady state rate was found to be completely inhibited (100%) with $\sim$ 62 μM Hg^{2+} concentration. The curve is single exponential in nature indicating monophasic inhibition of the enzyme. Low concentrations (60 μM) of Hg^{2+} ions were needed for 100% inhibition of the enzyme. This result is somewhat different from the inhibition by Cu^{2+} ions. It has been proposed recently that the interaction of Hg^{2+} with XO involves the S atom of the Mo(VI)=S moiety of the active centre (Rodrigues *et al* 1991). Since Hg^{2+} ion blocks the terminal S atom of the Mo=S moiety (Kramer *et al* 1987), which is catalytically important, we consider that the inhibition of the enzyme by this metal ion is primarily due to this interaction. The concentration of the Cu^{2+} ion required to reach the maximum inhibition (58%) of the enzyme was high (167 μM). But 48% inhibition was achieved during the first phase at only 30 μM concentration of Cu^{2+} ions. In the case of Hg^{2+}, a concentration of 62 μM of the metal ion was needed to achieve 100% inhibition. The inhibition of the first phase by Cu^{2+} and the total inhibition by Hg^{2+} are monophasic and require low metal ion concentrations. We suggest that the interactions operative during these situations are similar and may involve interaction of the S atom of the Mo=S moiety. The stability of the mercury sulphide bond is $\sim 10^{17}$ times higher than copper sulphide (Seidell *et al* 1952). Because of the very high stability of the Hg^{2+}–S bond, binding of this metal ion to the inorganic S atom of the Mo=S moiety is much stronger compared to that of the

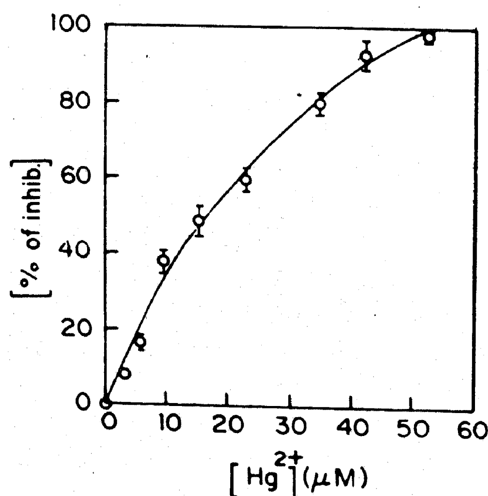

Figure 4. The dependence of the percentage of inhibition on Hg^{2+} ion, at pH = 7·2, the activity of the enzyme was measured as described earlier for figure 3.

Cu^{2+} ion. The higher inhibition of xanthine oxidase by Hg^{2+} (100%) compared to Cu^{2+} (58%) ion is therefore not unexpected.

4. Conclusions

XO has been isolated and purified in considerably good yields and in purified form following a slight modification of the reported procedure. In the first ammonium sulphate fractionation step, the required concentration of this salt may depend upon the amount of fat present in the cream. The inhibition of the enzyme activity by Cu^{2+} and Hg^{2+} ions has been investigated. The nature of the inhibition by Cu^{2+} ions was observed to be multiphasic, while that by Hg^{2+} ions was found to be monophasic. The inhibition by Hg^{2+} and the first phase of the inhibition by Cu^{2+} have been correlated to the interaction of the metal ions with the terminal S atom of the Mo=S moiety. The absence of any multiphasic inhibition in the case of Hg^{2+} ions may be due to its very high affinity towards the inorganic sulphur atom.

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