Mixed Inhibition of the Oxidoreductase Activity of Xanthine Oxidase by Pd²⁺ Ion Apurba Kumar Sau, Madhu Sudan Mondal and Samaresh Mitra*

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Pd²⁺ ion shows 100% inhibition of the oxidoreductase activity of xanthine oxidase, the nature of the inhibition being the uncommon mixed type (competitive–non-competive); to our knowledge this is the first example of mixed inhibition of xanthine oxidase, and also of the fact that Pd²⁺ can act as an inhibitor of enzyme activity.

Milk xanthine oxidase (XO) is a complex metallo-flavo enzyme which catalyses the oxidation of xanthine to uric acid in the presence of oxygen. It has two independent subunits each containing four redox centres: a molybdenum(vi) centre, two iron-sulfur (2Fe/2S) clusters Fe/S I and Fe/S II, and a flavin adenine dinucleotide (FAD) unit.1-3 Various small molecules are known to inhibit the activity of XO. It has been shown that metal ions such as Cu^{2+} and Hg^{2+} inhibit the oxidoreductase activity^{4,5} of this enzyme through non-competitive binding of the metal ions. We report here that Pd²⁺ ion can also inhibit the activity of this enzyme and the nature of inhibition belongs on the uncommon mixed type (i.e. competitive and non-competitive at the same time). To our knowledge this is the first report of mixed-inhibition of XO activity by an inhibitor as well as the capability of Pd²⁺ ion to act as an inhibitor of enzyme activity.

XO was isolated and purified by the method of Massey *et al.*⁶ The identity and purity of the enzyme was ascertained by optical spectra, AFR (=120; activity to flavin ratio) and PFR (=5.8; protein to flavin ratio) values. Analytical grade PdCl₂ and NaH₂PO₄ were used. Since PdCl₂ is not completely soluble in water, the concentration of PdCl₂ was measured by converting PdCl₂ to [PdCl₄]²⁻ by adding excess dilute HCl. The known absorption coefficients of XO (37 800 dm³ mol⁻¹ cm⁻¹ at 450 nm)⁸ and [PdCl₄]²⁻ (12 000 dm³ mol⁻¹ cm⁻¹ at 277.7 nm)⁷ were used for measuring their respective concentrations. All the experiments were performed on a Shimadzu UV-2100 spectrophotometer at pH 6.9.

The steady-state kinetics of the formation of uric acid from xanthine, catalysed by XO, were performed by monitoring the increase in absorbance at 295 nm. The enzyme activity was found to be inhibited by 100% at $\approx 200 \ \mu mol \ dm^{-3} \ Pd^{2+}$ ion concentration. The Michaelis constant (K_m) obtained from the Lineweaver–Burk (LB)⁸ double reciprocal plot at zero palladium concentration (data not shown) was found to be 5.0 $\mu mol \ dm^{-3}$ which is in good agreement with the literature value. Fig. 1 shows the LB plot of the steady-state inhibition



Fig. 1 The Lineweaver–Burk plot for the steady-state formation of uric acid from xanthine catalysed by XO. The formation of uric acid at 295 nm, monitored with increasing Pd²⁺ concentration at pH 6.9, was plotted as (initial rate)⁻¹ vs. [xanthine]⁻¹. The straight line plots are for 30 (\bigcirc), 60 (\bigtriangledown), 90 (\square), 150 (\diamondsuit) µmol dm⁻³ Pd²⁺ ion.

with increasing Pd²⁺ ion concentrations, which give straight lines meeting at a point between the horizontal and vertical axis. Mechanistically this type of inhibition has been interpreted to be of mixed type, where both competitive and noncompetitive interaction are responsible for the inhibition of the enzyme. The modified LB equation for this mixed inhibition is given by eqn. (1) where K_{m}' and V'_{max} are defined by eqns. (2) and (3).

$$\frac{1}{\nu} = \frac{1}{V'_{\max}} + \frac{K'_{m}}{V'_{\max}} \frac{1}{[Xan]_0}$$
(1)

$$K'_{\rm m} = \frac{K_{\rm m} \{1 + ([I_0]/K_{\rm i})\}}{\{1 + ([I_0]/K_{\rm I})\}}$$
(2)

$$V'_{\max} = \frac{V_{\max}}{\{1 + ([I_0]/K_I)\}}$$
(3)

v is the initial steady-state rate of the conversion of xanthine to uric acid. K_i and K_I are the inhibition constants due to competitive and non-competitive binding of Pd²⁺ to XO respectively. [I₀] is the total concentration of inhibitor, [Xan]₀ is the total concentration of xanthine, K_m is the Michaelis constant and V_{max} is the maximum velocity of the reaction of XO with xanthine. The slope (= K'_m/V'_{max}) in the LB plot of eqn. (1) can be written in terms of eqn. (4). The plot of I₀ vs.

$$\frac{V_{\max}K'_{m}}{V'_{\max}K_{m}} = 1 + \frac{[I_{0}]}{K_{i}}$$
(4)

the slopes of the lines obtained from Fig. 1 gives a straight line with an intercept of unity, which is consistent with eqn. (4) for mixed-inhibition [Fig. 2(*a*)]. From Fig. 2(*a*), we obtain $K_i = 42 \pm 8 \ \mu mol \ dm^{-3}$

The intercepts obtained from the straight lines of Fig. 1 can be represented by eqn. (5).

$$\frac{1}{V'_{\max}} = \frac{1}{V_{\max}} + \frac{[I_0]}{K_I V_{\max}}$$
(5)

Fig. 2(b) shows the plot of the intercepts $(1/V'_{max}) vs. [I_0]$, which again gives a straight line as expected from eqn. (5). The inhibition constant (K_I) obtained from this plot was 350 ±40 µmol dm⁻³.

The inhibition of XO activity by Cu²⁺ and Hg²⁺ ions has been found to give non-competitive inhibition (unpublished data). Similarly, the inhibition of XO by other small molecules is known to show only one type of inhibition. Hence, the present report is a unique example of the mixed inhibition of XO which indicates that native XO can form two inhibitory complexes with Pd2+ ion. Further, the non-competitive nature of the inhibition indicates that both the native and xanthinebound XO are capable of forming inhibitory complexes with Pd²⁺ ion. The values of the inhibition constants differ markedly, indicating that the nature of the coordination of the Pd²⁺ ion for the two binding sites is different. The binding of Pd²⁺ for the competitive inhibition is expected to take place near the xanthine binding region (molybdenum centre) of XO. The lower value of the inhibition constant for the competitive inhibition as compared to the non-competitive inhibition indicates a higher coordinating ability of the donor atoms to the Pd²⁺ ion near the molybdenum centre.

Inorganic palladium complexes have been observed to show

(a)

(b)

6.0

4.0

2.0

0.8

0.6

0.4

from the straight lines of Fig. 1.

V_{max} K^{''}_{max} K_m

Ľ.

limited biological activity due to the high lability of palladium-(II).^{13,14} However, in the present case Pd²⁺ was found to inhibit the XO activity indicating the formation of inhibitory

180 0 45 90 135 $[Pd^{2+}] / \mu mol dm^{-3}$ Fig. 2 A plot of $V_{\text{max}} K'_m / V'_{\text{max}} K_m vs. [Pd^{2+}](a)$. The value of V_{max} / K_m (=1.8) was calculated from the LB plot of the steady-state reaction in the absence of the inhibitor. The plot of the intercepts (int.) vs. $[Pd^{2+}]$

is shown in (b). The intercepts $(dm^3 min^{-1} \mu mmol^{-1})$ were obtained

complexes with the enzyme, which may be of interest in general.

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