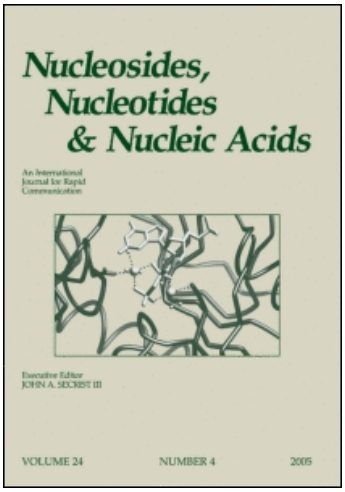

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Kinetic Model of Oxidation Catalyzed by Xanthine Oxidase—The Final Enzyme in Degradation of Purine Nucleosides and Nucleotides

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KINETIC MODEL OF OXIDATION CATALYZED BY XANTHINE OXIDASE—THE FINAL ENZYME IN DEGRADATION OF PURINE NUCLEOSIDES AND NUCLEOTIDES

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▫ *A new kinetic model is presented for analysis of experimental data of oxidation process catalyzed by milk xanthine oxidase. The kinetics for two substrates, xanthine and its analog 2-chloroadenine, in a broad pH range (5.8–9.0) are best described by an equation which is a rational function of degree 2:3 and 2:2, respectively.*

Keywords Xanthine Oxidase, Enzyme Kinetics, Purine Degradation, Biological Oxidation, Non-Michaelis–Menten Kinetics

INTRODUCTION

Xanthine oxidase (XO) is a molybdeno-flavoprotein complex involved in the final stage of degradation of nucleic acids' components. It catalyzes the oxidation of hypoxanthine (Hx) to xanthine (Xan) and subsequently to uric acid that is the end product of purine catabolism in man.[1] Accumulation of uric acid in human blood plasma (hyperuricemia) leads to formation and deposition of sodium urate microcrystals in joints, which results in gout.[2] Allopurinol, an analog of Hx and a weak XO inhibitor developed 30 years ago, is the only clinically used drug for the treatment of hyperuricemia associated with chronic gout.[3]

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XO seems to play an important role that is not limited to oxidation pathway in purine metabolism. It was suggested that the enzyme is involved in pathogenesis of post-ischemic reperfusion tissue injury and in protection against bacterial infection, toxic plants and metabolic xenobiotics by producing reactive oxygen species during the redox cycle.[4,5] Serum XO is significantly elevated in various pathological states like hepatitis, inflammation, malignancies and ageing, and it is commonly accepted that reactive oxygen species generated by the enzyme are involved in oxidative damage. Thus, the inhibition of this enzymatic pathway could be beneficial in a broad spectrum of pathologies, and not only in treatment of gout.[6]

XO-catalyzed oxidation of biological substrates (Hx and Xan), and inhibition of the enzyme by compounds with potential medical applications, have been described in the literature using the Michaelis–Menten equation. However, as outlined by Bardsley et al.,[7] experimental data obtained even for narrow ranges of substrate concentration showed pronounced deviation from the hyperbolic kinetics. Hence, interpretation of experimental data from XO-catalyzed processes in terms of classical parameters (K_m, V_{max} for substrates, and K_i for inhibitors) is inappropriate.

Another important problem that has been overlooked in the studies of XO is the possible role of ionic forms of purine substrate in interactions with the enzyme and in catalysis. Xan has a pK_a of 7.7, which is attributed to ionization of N-3 position. Hence, in many physiological and pathological conditions there is a mixture of anionic and neutral forms of the substrate in solution.[8,9] This should be taken into account when analyzing kinetic data and constructing kinetic and molecular models of the XO catalytic mechanism.

EXPERIMENTAL

Xanthine oxidase from bovine milk (EC 1.2.3.2) and xanthine were purchased from Sigma (St. Louis, MO). 2-chloroadenine (2ClAde) was synthesized according the procedure described previously.[10] Concentrations of the enzyme and substrates in solutions were determined from absorbance data using the respective molar extinction coefficients. For XO ε_{max}(280 nm) = 202 600 $M^{-1}cm^{-1}$ was determined based on the amino acid sequence P80457 obtained from SwissProt database. For Xan ε_{max}(267 nm) = 10,300 $M^{-1}cm^{-1}$ at pH 6.0, ε_{max}(270 nm) = 9,100 $M^{-1}cm^{-1}$ at pH 7.5, ε_{max}(276 nm) = 9,300 $M^{-1}cm^{-1}$ at pH 9.0; for 2ClAde ε_{max}(264 nm) = 12,000 $M^{-1}cm^{-1}$ at pH 6.0, ε_{max}(264 nm) = 11,900 $M^{-1}cm^{-1}$ at pH 7.5, ε_{max}(266 nm) = 11,100 $M^{-1}cm^{-1}$ at pH 9.0. Due to low solubility, 2ClAde samples were first dissolved in a slightly alkaline medium (0.01 NaOH) and then diluted with a phosphate buffer of the appropriate pH value.

Enzymatic reactions have been performed at 25°C in 50 mM phosphate buffer in the pH range 5.8–9.0. Ultraviolet absorption spectra were recorded with an Uvikon 940 UV-VIS spectrophotometer (Kontron, Vienna, Austria), fitted with thermostatically controlled cell compartments. Oxidation of Xan was monitored

at 300 nm corresponding to the uric acid absorption. Formation of 8-hydroxy-2-chloroadenine, the oxidation product of 2ClAde, was monitored at 285 nm. Initial velocity of both processes was calculated using linear regression of absorbance *vs.* time.

Nonlinear regression analysis of the experimental data was performed using GraphPad Prism 3.0 (GraphPad Software Inc.) and Origin 6.0 (Microcal) computer software packages. Rational functions of degree 2:2, 2:3 (Eqs. 1 and2), 3:2 and 3:3 were fitted[7] to kinetic data of initial velocity (v_o) *vs.* initial substrate concentration (c_o). Statistically significant decrease in the sum of residuals (as estimated with the Snedecor's F test at a 95% confidence level), residual plots, and P value from the runs test were used to identify major deviations from the equation selected, and to discriminate between various models.

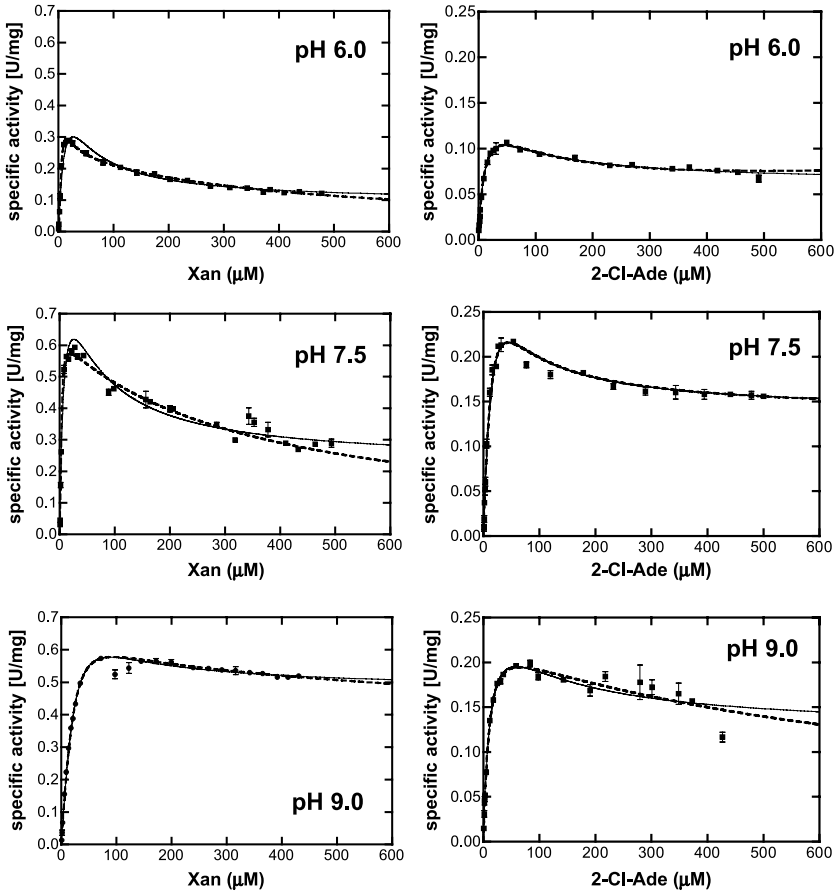

FIGURE 1 Kinetic of oxidation of Xan (left panels) and 2CldAde (right panels) catalyzed by XO. Reactions were conducted at 25°C in 50 mM phosphate buffer at pH values indicated. Fitting of the rational function of degree 2:2 (Eq. 1) and of degree 2:3 (Eq. 2) to the initial velocity data $v_o(c_o)$ led to solid and broken lines, respectively.

RESULTS AND DISCUSSION

Our aim was to develop a suitable kinetic model for mammalian XO-mediated catalysis by studying the oxidation process of the natural substrate Xan and its structural analog 2ClAde over a wide range of the initial substrate concentrations (0.5–500 μM) and in a broad range of pH values (5.8–9.0). The pK_a = 7.7 of Xan deprotonation indicates that both in our experiments and in biological conditions there is a mixture of the neutral and monoanionic forms of Xan. The situation is quite different for 2ClAde. The pK_a of 8.5 for this compound, which value was determined by spectrophotometrical titration, indicates that 2ClAde exists predominantly in the neutral form in the majority of biological environments.

The initial velocity data $v_o(c_o)$ obtained for both compounds at pH 6.0, 7.5, 9.0 are presented in Figure 1. (Data obtained at pH 5.8, 6.5, 7.0, 8.0 and 8.5 are not shown). Kinetics at all pH values studied is clearly non-hyperbolic, hence the use of the Michaelis–Menten equation is not adequate. The data could be described as an apparent substrate inhibition, since maximal velocity is obtained for a relatively low initial substrate concentration (20–80 μM, depending on pH and substrate employed), and the reaction rates are lower for higher c_o. The rates of oxidation are pH dependent. The maximum velocity is about 0.3, 0.6, and 0.6 U/mg for Xan, and 0.1, 0.2, and 0.2 U/mg for 2ClAde for pH 6.0, 7.5, and 9.0, respectively. Hence, pK_a of the substrate appeared to not influence the pH dependence of the maximal rate of oxidation. However, substrate concentration corresponding to the maximal rate is pH dependent for Xan, and pH independent for 2ClAde (Figure 1), suggesting that the neutral form of Xan binds stronger to XO than the monoanion.

Nonlinear regression analysis of the kinetic data yielded adequate fits for rational functions of degree 2:2 for 2ClAde (Eq. 1) and 2:3 for Xan (Eq. 2, see Figure 1):

$$v_o(c_o) = \frac{jc_o + ic_o^2}{1 + mc_o + lc_o^2} \tag{1}$$

$$v_o(c_o) = \frac{jc_o + ic_o^2}{1 + mc_o + lc_o^2 + kc_o^3} \tag{2}$$

These are the mathematical expressions describing several reaction mechanisms; constants i, j, k, l, and m represents combination of rate constants and their interpretation depends on the molecular mechanism.

Hence, in general, a rational function of degree at least 2:2 is necessary to describe kinetic data for XO. Such a function describes several possible molecular mechanisms of enzymatic reactions, e.g., allosteric interactions between enzyme subunits, steady-state random systems and many others (e.g., Refs. [7,11,12]). Steady-state kinetic data alone does not permit to distinguish unequivocally between various mechanisms. However, it is clear from the data presented here that

interpretation of kinetic data for XO on the basis of Michaelis–Menten model is incorrect. This should be given special consideration when describing new inhibitors of XO with potential medical applications. Determination of classical inhibition types and inhibition constants (K_i) is not feasible for XO inhibition and may lead to incorrect conclusions regarding possible clinical application of candidate compounds. In this case, IC_{50} values seem to be more adequate to characterize inhibitory potency of new and previously synthesized analogues.

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