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단 신

변형된 퓨린유도체를 이용한 Xanthine oxidase의 반응 메카니즘 연구

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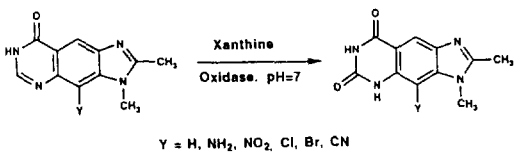
Modified Purines as Mechanistic Probes of Substrates Oxidation by Xanthine Oxidase

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Xanthine oxidase oxideze a variety of substrates such as hypoxanthine, pteridine and pyrimidines. The finding by Leonard *et al.*[1] that benzo separated hypoxanthine is oxidized at an apparent faster rate than hypoxanthine itself, indicate that steric interference is not critical factor to the rate of oxidation. In other words, ready conversion of benzo-separated hypoxanthine into benzo separated xanthine shows that active site of xanthine oxidase can accept substrates larger than hypoxanthine. The catalytic mechanism of oxidation is thought to involve hydride transfer (or its equivalent) from the substrate in concert with nucleophile transfer to the substrate[2]. The mechanism has been proposed by Edmondson *et al.* Skibo *et al.*[3~5]. Attacking nucleophile has been proposed to be a terminal oxo group on the molybdenium center by Gutteridge and Bray[6,7]. In spite of various studies, the detailed mechanism of nucleophile assisted substrate oxidation was reported recently[8,9]. So it will be worthwhile to verify the proposed mechanism and study the structure of active site by doing substrate oxidation using electronically modified substrates.

In order to probe the mechanistic process by means of Hammett plot, various 4-substituted imidazo[4,5-g]quinazoline derivatives were synthesized (*Scheme* 1). All these benzo-separated compounds showed excellent substrate activity of xanthine oxidase and subsequent oxidation of 6-position gave corresponding benzo separated uric acid analogues. The substrate specificity were comparable with natural substrates too.



Scheme 1

It is expected that there might be profound changes in catalytic parameters as electronic structure of the substrate is changed. But as mentioned earlier, xanthine oxidase active site can tolerate dimensionally altered substrates. It suggests that the substrate oxygen reductase activity of imidazol[4,5-g]quinazoline bearing substituents in 4-position would be influenced mainly by the electronic factor of the substituents rather than steric factor. As described previously[3]. The electronic effect reflected in basicity and nucleophilicity. Thus electronic effect can change the catalytic parameters by changing the rate of nucleophilic attack on the substrate during oxidation. In fact, this assumption has already proved to be incorrect and the Hammett plot obtained for substrate-oxygen

reductase activity is scattered. So it is reasonable to assume that changing the substrates-oxygen reductase activity is caused by basicity difference in the substrates. The relationship between catalytic parameters and electronic effect represented as basicity will provide insight into the reaction pathway. Also, kinetic isotope effects of 6-deuterated derivatives will provide evidence of hydride transfer in the rate determining step.

Due to the difficulties arising from the synthesis of 4-substituted substrates, only few substrates such as 4-nitro, 4-bromo and 4-chloro were examined as substrates of buttermilk xanthine oxidase with 0.027~0.27 micromol of enzyme in 0.05 *M* pH 7.40 phosphate buffer (ionic strength=1.0, KCl) at 30± 0.2℃. All compounds were quantitatively oxidized to the respective 6-oxo derivatives as verified by observing the disappearance of 6-proton chemical shift in NMR. Lineweaver-Burk plots for the substrate-oxygen reductase activity indicate that the substrates are capable of inhibiting the enzyme at high concentrations. The apparent values of K_m and K_{cat} for the substrate-oxygen reductase activity were obtained from Lineweaver-Burk plots of low substrate concentrations. Xanthine also inhibits the enzyme when present at high concentrations, probably as a result of the formation of an inactive substrate enzyme substrate complex. The presence of such complex suggests that the apparent values of K_m and K_{cat} contain binding constants not involved in product formation. Thus the observed changes in these parameters with electronic character of the substituent could be due to change in productive as well as nonproductive constants. Indeed, a Hammett plot obtained with K_{cat} values exhibits a high degree of scattering as described previously[3].

In order to focus exclusively on equilibria and first order processes involved in product formation, the plot was obtained with the ratio, of apparent K_{cat}/K_m. This ratio is unaffected by both nonproductive binding and the accumulation of intermediates, and it is equal to the product of equilibrium and first order constants involved in product formation. Found in *Fig.* 1 is the Hammett plot

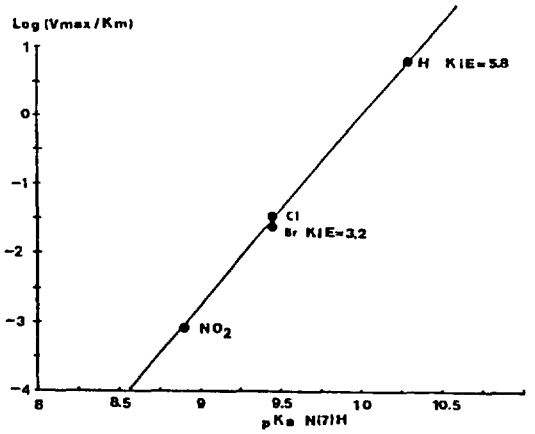


Fig. 1. Plot of log (V_{max}/K_m) *vs.* pK_a of N (7)-H of substrates. The catalytic parameters are obtained in pH 7.40 phosphate buffer (μ=0.1, KCl) at 30℃.

for the oxidation of imidazo[4,5-g]quinazoline derivatives as substrates. The pK_a values for acid dissociation from the pyrimidine N(7)-H were used in place of sigma values for determining the electronic effects of substituents. The use of sigma values is not reliable since the nitro and amino substituents are probably not planar with ring plane. So, there might be no through resonance effect to the reaction center by substituents. A plot of log (V_{max}/K_m) *vs.* pK_a of the N(7)-proton and kinetic isotope effects are also shown in *Fig.* 1.

The V_{max}/K_m term has the dimension of a first order rate constants and is the product of enzyme concentrations, rate constants on the reaction path and equilibrium constants on the reaction path as shown previously[7]. The KIE values are based on the relative values of V_{max}/K_m for the 6-H and 6-D derivatives. The positive slope of the plot indicates that a negative charge is being released from the substrate in rate determining step. The KIE on the plot indicates that hydride (or its equivalent) transfer is occurring in the rate determining step. The important thing is that negative charge distribution arising from N(7)-H dissociation has exactly the same distribution as the one arising from the acid dissociation. Acid dissociation of 4-nitro derivative possesses a low pK_a (8.90) as a result of anion stabilization of substi-

tuent. Thus anionic species of 4-nitro derivative will be also stabilized, which means less tendency to give up hydride (or its equivalent). On the other hand, the 4-amino derivative has higher pK_a (10.33) and will give up hydride (or its equivalent) because of the lack of anion stabilization. The anionic form of 4-amino derivatives is present less than 3% at pH 7.40 and is likely not contributing to the changes in the catalytic parameters with pK_a. This means that all reactions are considered to involve the neutral substrate. But the use of apparent K_{cat}/K_m values would cancel out any acid dissociation terms in the individual catalytic constants. The amino substituted derivative possesses a much lower V_{max}/K_m value than the X=H and deviate little bit from the plot. This may be due to rate limiting nucleophile transfer resulting from the electron rich nature of substrate.

Inspection of *Fig.* 1 reveals that Hammett plot of substrates with pK_a 10.5>pK_a>8.5 shows linear relationship with a slope of approximately+2.6. Kinetic isotope effect obtained from the 4-bromo and 4-H substituents has relatively large values which might suggest that hydride transfer from the substrate to enzyme is the rate determining step in the oxidation. In the mechanistic point, fast persulfide attack followed by rate determining hydride (or its equivalent) transfer and hydrolysis of the sulfur-carbon bond is feasible. The deviated amino substituent is not shown in the plot. It possesses much lower V_{max}/K_m value than the X=H and thus deviates from the plot. As mentioned earlier, this behavior supported by the observation that various 6-substituted quinazolines as electronic probes of xanthine oxidase. It may indicate the change in the rate limiting step depending on the nature of substituents.

It is concluded that oxidation involved nucleophile transfer to the C(6)-center in concert with hydride (or its equivalent) transfer to the molybdenium center. Thus nucleophile increases the electron density in the substrates and thereby facilitate the hydride (or its equivalent) transfer. But for the further detailed assessment for the mechanism of the oxidation, it will be necessary to do additional work with variety of 4-substituted derivatives. Currently, we are attempting to synthesize 4-methoxy, 4-cyano, 4-methyl and 4-acetamido derivatives to access whether there is mechanistic change on the oxidation or not.

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